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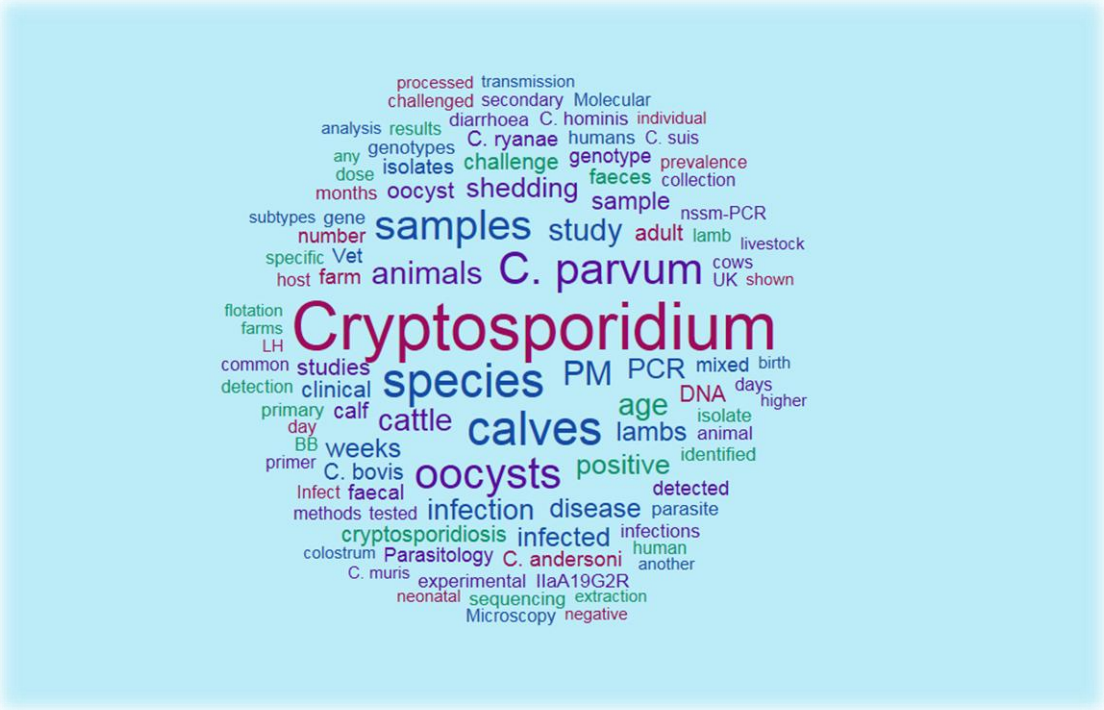
Cryptosporidiosis in Farm Livestock

Sarah Thomson



Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy, Institute of Biodiversity Animal Health and Comparative Medicine University of Glasgow 2016

Abstract

Although diarrhoea caused by *Cryptosporidium* is prevalent in livestock species throughout the world relatively little is known about the species and subtypes of *Cryptosporidium* found in cattle on Scottish farms. In particular, little is known about the shedding profiles (age when calves become infected and duration of shedding) of the different species found in cattle and how calves become infected. There are several theories about how neonatal calves first become infected with the parasite but the role which adult cattle play in the transmission of the parasite has not been fully addressed. It was previously thought that adult cattle did not become infected with the same species of *Cryptosporidium* which causes disease in the young calves. Some studies have shown that this may not be true and with the advance of new techniques to discriminate species this is an area which should be revisited.

In addition, it is known that it is possible for humans to become infected with *Cryptosporidium* and show clinical disease early in life and then again later in adulthood. In livestock however, diarrhoea caused by the parasite is generally only seen in neonatal livestock while older animals tend to be asymptomatic. It is not known if this resistance to clinical disease at an older age is due to changes in the host with an increase in age or if prior infection "immunises" the animal and provides protection against re-infection. It is also not known if infection with one isolate of *C. parvum* will provide protection against infection with another or if the protection formed is species/isolate specific.

The main aims of this thesis were to: determine the species and subtypes of *Cryptosporidium* found in calves on a study farm over a one year period from birth; assess the role which adult cattle play in the transmission of the parasite to newborn calves; develop new typing tools to enable the rapid and easy differentiation of *Cryptosporidium* species found in cattle and to examine the host-pathogen interactions in animals given serial experimental challenges with distinct *Cryptosporidium parvum* isolates to determine if the resistance seen in older animals on farms is due to an increase in age or as a result of prior infection.

A variety of different approaches were taken to achieve these aims. Longitudinal experiments carried out on a study farm revealed that in calves <9 weeks of age the most common species of *Cryptosporidium* is *C. parvum* and that all calves in the group became infected with *Cryptosporidium* within the first two weeks of life. Sample collection from the same animals later in life (at 6 months of age) showed that contrary to most previous studies the most common species detected at in this age group was also *C. parvum* although, interestingly, the subtype which the calves were shedding was not the same subtype that they were shedding previously.

The longitudinal study which investigated the role of adult cattle in the transmission of *Cryptosporidium* also yielded some interesting results. It was found that most of the adult cattle on this farm were shedding *Cryptosporidium* albeit intermittently. Speciation of the positive samples revealed that, on this farm, the most predominant species of *Cryptosporidium* in adult cattle was also *C. parvum*. This is very unusual as most previous studies have not found this level of infection in older cattle and *C. parvum* is not usually found in this age group. A number of different subtypes were found in adult cattle and some animals shed more than one subtype over the course of the study. This contradicts prior findings which demonstrated that only one subtype is found on a single farm.

The experimental infection trial involving infection of young (<1 week old) and older (6 week old) lambs with distinct *C. parvum* isolates demonstrated that an increase in age at primary infection reduces the effect of clinical disease. Animals which were infected at <1 week of age were re-challenged at 6 weeks of age with either a homologous or heterologous infection. Results revealed that previous exposure does not protect against re-infection with the same or a different isolate of *C. parvum*. This study also demonstrated that an increase in infective dose leads to a shorter pre-patent period and that there are variations in the clinical manifestations of different isolates of the same *Cryptosporidium* species.

Table of contents

List of Tables

List of Figures

Acknowledgements

Firstly, I would like to thank my supervisors at the Moredun Research Institute, Dr. Frank(tastic) Katzer and Prof. Lee Innes very much for all their support and guidance over the past three and a bit years. You have been wonderful supervisors and it has been a pleasure working with you. Also many thanks to Prof. Nick Jonsson my University supervisor, particularly for help with statistical analysis. I am extremely grateful for all of the time and advice all of my supervisors have given me even when I am sure they were busy with more important things. Thank you also for enabling me to attend so many conferences and meetings to share my research with the world ☺

I have thoroughly enjoyed my time at the Moredun Research Institute, it is a very friendly and fun place to work. I need to thank the Proto & Co team for making my experience here so positive. Thank you also for helping me with the huge lamb study, from making little poo bags and harnesses to feeding lambs and collecting samples. A special thank you to Alessandra Toroda for spending months in the lab making poo-smoothies and counting oocysts, to Dr. Emily Hotchkiss for assisting with my stats understanding and to Dr. Beth Wells for all the additional help she gave me above and beyond what was necessary.

I am very fortunate that my work colleagues are also my friends so I must thank them too, in particular Laura Doull for coming to pole with me so that we had something other than our PhD's to ~~complain~~ talk about. To Stefano Guido, Beth Wells, Marieke Opsteegh, Maria Parigi and Alessandra Toroda for being wonderful office mates and (sometimes) joining me in a spot of office yoga to relieve the stresses of the day as well as putting up with the general chaos that I cause (I WILL tidy my desk soon, I promise).

Finally I need to thank my family and friends from outside of work for their support and understanding over the course of my PhD and previous studies. I am sure that you all know at lot more about *Cryptosporidium* than you ever wanted to! A big thank you to Alistair who has listened with patience to my presentations and for offering constructive advice and just generally putting up with me ☺

Author's Declaration

I declare that the work presented in this thesis is my own work unless stated. This work has not been previously submitted for any other degree or professional qualification.

Sarah Thomson

Definitions/Abbreviations

%	Percent
$(NH_4)_2SO_4$	Ammonium Sulphate
~	Approximately
<	Less Than
=	Equal To
>	More Than
°C	Degrees Centigrade
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
ACVM	aniline-carbol-methyl-violet
AF	Acid Flocculation
AI	Artificial Insemination
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of Variance
AP	Auramine Phenol
APC	Antigen Presenting Cell
BB	*C. parvum* isolate BB
bp	base pairs
BSA	Bovine Serum Albumin
CD4+ T	T-helper cell expressing cluster of differentiation 4 glycoprotein
CE	Capillary Electrophoresis
COWP	*Cryptosporidium* Oocyst Wall Protein
d	Day
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleic Triphosphate
DPI	Days Post-Infection
EDTA	Ethylenediaminetetraacetic Acid

EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
EtOH	Ethanol
FAM	Carboxyfluorescein
fg	Femtogram
FITC	Fluorescein Isothiocyanate
g	grams
g	Gravitational Force
GP60	60-kDa glycoprotein
gp60	60-kDa glycoprotein gene
h	hour
HBSS	Hanks Buffered Saline Solution
HEX	Hexachlorofluorescein
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSP70	Heat Shock Protein 70
ID_{50}	Infectious Dose to 50% of Exposed Individuals
IFAT	Immunofluorescence Antibody Test
IFN-γ	Interferon-gamma
IL	Interleukin
IMS	Immunomagnetic Separation
Isolate	*C. parvum* isolate which is derived from naturally infected calves, differs at more than one loci and which differs in clinical manifestation
kDa	Kilo Dalton
kg	Kilogram
l	Litre
LH	*C. parvum* isolate LH
mg	Milligram
$MgCl_2$	Magnesium Chloride
mins	minutes
ml	Millilitre
MLG	Multilocus Genotype
MLST	Multilocus Sequence Typing
mm	Millimetre

mM	Millimolar
mZN	Modified Ziehl-Neelsen
n	Number
ng	Nanogram
NGS	Next Generation Sequencing
NK	Natural Killer
nssm	Nested Species Specific Multiplex
opg	Oocysts per gram
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Negative log of the activity of the hydrogen ion in an aqueous solution
pmol	Pico mol
qPCR	Quantitative PCR
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribonucleic Acid
s	Second
SDS	Sodium Dodecyl Sulphate
SF	Salt Flotation
spp.	Species
SSU	Small Subunit
TE	Tris(hydroxymethyl)aminomethane-Ethylenediamintetraacetic Acid
TNFα	Tumor Necrosis Factor Alpha
TNFß	Tumor Necrosis Factor Beta
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloride
UHT	Ultra High Temperature
UK	United Kingdom
USA	United States of America
UV	Ultra-Violet
vs.	Versus
wk	Week
wk1	Infected at <1 week of age
wk6	Infected at 6 weeks of age

Chapter 1: General Introduction

1.1 Background

Cryptosporidiosis is the disease caused by a protozoan parasite of the genus *Cryptosporidium,* which was discovered in 1910 by Edward Ernst Tyzzer in the gastric glands of mice. Tyzzer noticed that this parasite did not contain sporocysts within the oocysts and it sporulates while still attached to the host wall. For this reason Tyzzer named the genus *Cryptosporidium,* from the Greek kruptos meaning 'hidden', and the parasite that he identified in the gastric glands, *Cryptosporidium muris.* Three years later Tyzzer identified another species of *Cryptosporidium* which was not infective in the gastric glands of mice but only in the small intestine (Tyzzer, 1910). This second species produced oocysts which were smaller in size (4-5 µm) compared with *C. muris* oocysts (6-8 µm), Tyzzer named this species *Cryptosporidium parvum* (*parvum* comes from the Latin for little). It was originally believed that there were only two different species of *Cryptosporidium* but there are now over 27 described species and many more genotypes (Chalmers and Katzer, 2013).

Cryptosporidiosis, the disease caused by some species of *Cryptosporidium* in susceptible hosts generally manifests itself as profuse watery diarrhoea, weight-loss and inappetence. Depending on the parasite and host species other clinical signs may be seen such as vomiting and nausea in some humans infected with *Cryptosporidium hominis.* Despite being identified in many animal species, cryptosporidiosis was not thought to be a very important disease until the early 1980s and the HIV-AIDS epidemic, though it was first reported in humans in 1976 (Nime et al., 1976). Of the first seven reported cases of *Cryptosporidium* infection in humans, six were from immunocompromised patients leading to the belief that if the immune system was not compromised, cryptosporidiosis would not pose any health risk (Bird and Smith, 1980). Of course cryptosporidiosis is now recognised as an important zoonotic disease, which affects many species (Chalmers and Katzer, 2013).

Cryptosporidium was first associated with diarrhoea in calves in 1971 (Panciera et al., 1971) and since then has been identified as one of the most important

cause of neonatal enteritis in calves worldwide (de Graaf et al., 1999; Fayer et al., 1998; Thompson et al., 2005).

1.2 Life Cycle

Cryptosporidium is a monoxenous organism, (life cycle completed within a single host) which exists in the environment in thick walled oocysts. These oocysts are very resistant to many environmental factors such as high and low temperatures (Fujino et al., 2002) but they can be inactivated by desiccation (Robertson et al., 1992). *Cryptosporidium* oocysts survive well in cool, moist climates and can remain infective for many months (Robertson et al., 1992).

Infective *Cryptosporidium* oocysts (Figure 1-1(a)) are ingested by the host and when triggered by the conditions in the gastrointestinal tract (low pH, body temperature) excystation occurs (Hijjawi et al., 2002) and four sporozoites are released (Figure 1-1(b)). The sporozoites attach themselves to the host epithelial cells at the ileocaecal junction which they then invade and become engulfed by the cells forming a parasitophorous vacuole. Many other protozoan parasites also exist within a parasitophorous vacuole but *Cryptosporidium* also contains a unique structure known as the feeder organelle, which separates the cell and parasite cytoplasm (Pohlenz et al., 1987).

After the development of the feeder organelle the sporozoite itself becomes more spherical in shape and forms a trophozoite (Figure 1-1 (c)). The parasite begins asexual reproduction and develops into a Type I meront (Figure 1-1 (d)) or a Type II meront (Figure 1-1 (f)). Merozoites are formed within the Type I meront, each Type I meront releases 6-8 merozoites which can immediately re-infect the host, by invading neighbouring cells and beginning asexual reproduction again, or develop into a Type II meront (Figure 1-1 (f)).

Type II meronts release four merozoites which initiate the sexual reproductive cycle (Figure 1-1 (f)). The released merozoites invade host cells and differentiate into either macrogamonts (Figure 1-1 (h)) or microgamonts (Figure 1-1 (g)). Microgamonts develop multi-nuclei and release free microgametes which penetrate and fertilise the macrogamete, producing a zygote (Figure 1-1 (i)). Meiosis occurs and the zygote differentiates into four sporozoites as the

oocyst develops and is released from the lumen. The sporozoites may be released directly into the lumen from thin-walled oocysts and re-infect the host (Figure 1-1 (k)) or thick-walled oocysts which are shed in faeces and remain immediately infective for other hosts (Figure 1-1 (j)).

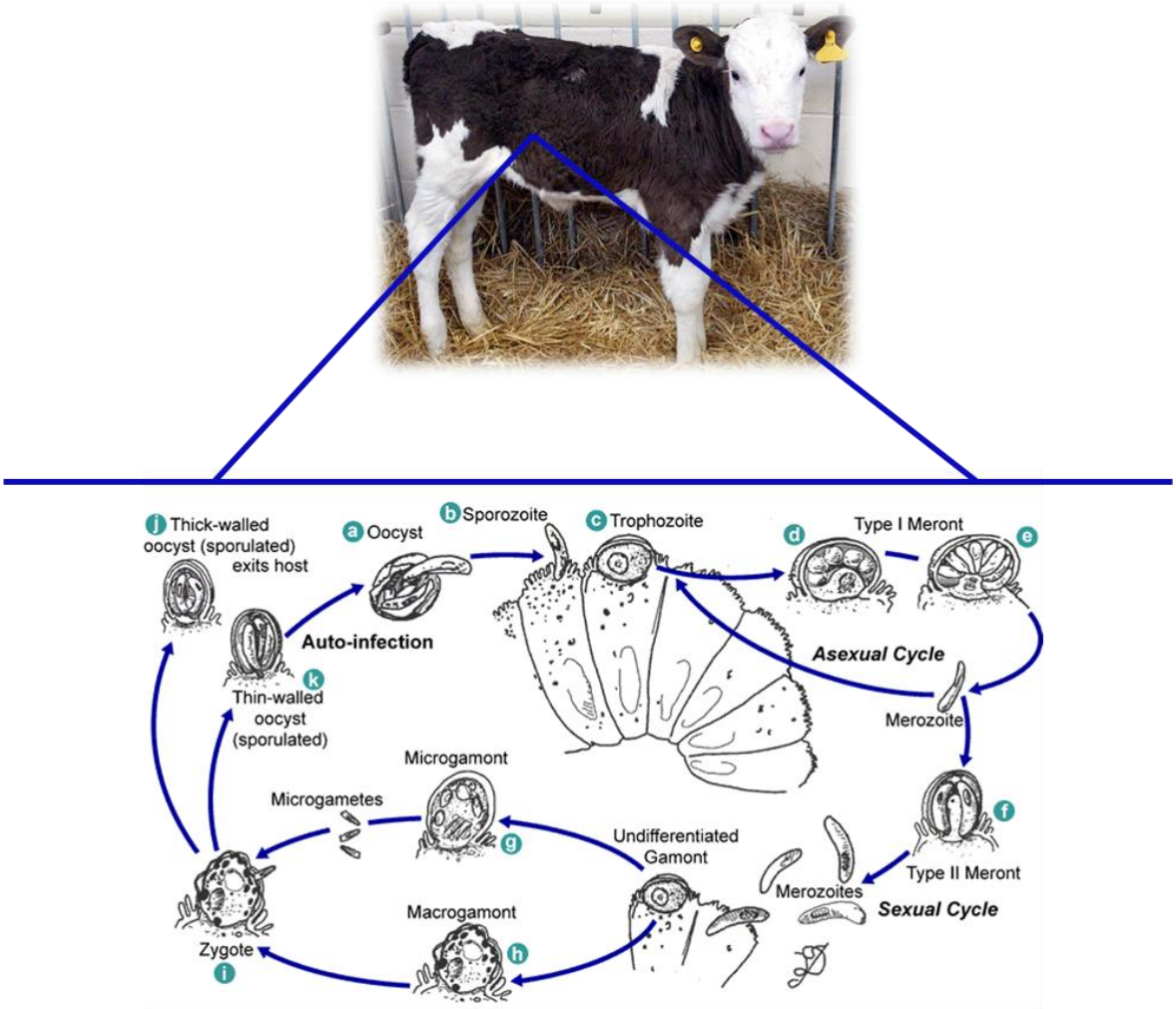


Figure 1-1: Life cycle of *Cryptosporidium*
Reproduced from Juranek DD. Cryptosporidiosis. In: Strickland GT, editor. Hunter's Tropical Medicine and Emerging Infectious Diseases, 8th ed. Philadelphia: WB Saunders; 2000. Originally adapted from the life cycle that appears in Current WL, Garcia LS. Cryptosporidiosis. Clinical Microbiol Rev 1991; 4:325-58.

1.3 Species of *Cryptosporidium*

There are 28 different species of *Cryptosporidium* currently recognised (Table 1-1) and many more genotypes (Chalmers and Katzer, 2013; Fayer, 2010). The most common species found in the UK and worldwide is the zoonotic species *C. parvum* (Chalmers and Katzer, 2013). Historically species of *Cryptosporidium* were named according to the host of origin on the assumption that most species were host-specific. While many of the species of *Cryptosporidium* do still appear to be host-specific others infect many different host species, for example, *C. parvum* and *C. ubiquitum* infect a wide variety of hosts.

Table 1-1: Currently recognised *Cryptosporidium* species and the hosts they infect

Species Name	Synonym	Host Species	First Described By
C. andersoni*	Mouse genotype	Cattle	(Lindsay et al., 2000)
C. baileyi		Birds	(Current et al., 1986)
C. bovis	Genotype Bovine B	Cattle	(Fayer et al., 2005)
C. canis*	Canine genotype	Dogs	(Fayer et al., 2001)
C. cuniculus*	Rabbit genotype	Rabbit	(Robinson et al., 2010)
C. ducismarci		Tortoise	(Traversa, 2010)
C. fayeri	Opossum genotype 1	Marsupials	(Ryan et al., 2008)
C. felis*		Cats	(Iseki et al., 1989)
C. fragile		Toads	(Jirku et al., 2008)
C. galli	*C. blagburni*	Birds	(Ryan et al., 2003b)
C. hominis*	Genotype 1/genotype H	Humans	(Morgan-Ryan et al., 2002)
C. huwi		Fish	(Ryan et al., 2015)
C. marcopodum		Marsupials	(Power and Ryan, 2008)
C. meleagridis*		Birds	(, 1955a)
C. molnari		Fish	(Alvarez-Pellitero and Sitja-Bobadilla, 2002)
C. muris*		Mice	(Tyzzer, 1910)
C. parvum*	Genotype 2/genotype C	Mammals inc. humans	(Tyzzer, 1912)
C. ryanae	Deer-like genotype	Cattle	(Fayer et al., 2008)
C. scophthalmi		Fish	(Alvarez-Pellitero et al., 2004)
C. scruforum		Pigs	(Kvac et al., 2013)
C. serpentis		Snakes	(Levine, 1980)
C. suis*	Pig genotype 1	Pigs	(Ryan et al., 2004)
C. tyzzeri	Mouse genotype 1	Mice	(Ren et al., 2012)
C. ubiquitum*	Cervine genotype	Variety of animals	(Fayer et al., 2010b)
C. varanii	*C. saurophilum*	Lizards and Snakes	(Pavlasek and Ryan, 2008a)
C. viatorum		Humans	(Elwin et al., 2012b)
C. wrairi		Guinea Pigs	(Vetterling et al., 1971)
C. xiaoi	*C. bovis*-like genotype	Sheep	(Fayer and Santin, 2009)

*also infects humans

1.3.1 Species of *Cryptosporidium* in farm livestock

Of the 27 or so *Cryptosporidium* species which have been identified about eight of these are known infect farm livestock (cattle, sheep and pigs).

1.3.1.1 Cattle

There are four species of *Cryptosporidium* which are commonly found in cattle; *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni*. There are some reports of other species also being found; *C. hominis* has been reported in a 3 day old calf and a 6 year old cow from Scotland (Smith et al., 2005), *C. suis* in a calf (Fayer et al., 2006) and *C. felis* in a cow (Bornay-Llinares et al., 1999) but these reports are rare.

1.3.1.2 Sheep and Goats

Sheep and goats are predominantly infected with *C. parvum, C. xiaoi, C. bovis* and *C. ubiquitum* although rare occurrences of other species have been reported. *Cryptosporidium hominis* has been reported as the predominant species in sheep on the Scottish island of St Kilda (Connelly et al., 2013), in a sheep and a goat kid from a farm and petting zoo in the UK (Giles et al., 2009) and in a sheep in Australia (Ryan et al., 2005). *Cryptosporidium scruforum* and *C. andersoni* have been detected in sheep from flocks in Australia (Yang et al., 2014) and *C. fayeri* has been reported in a sheep (Ryan et al., 2005) again these occurrences of other species are rare.

1.3.1.3 Pigs

The predominant species of *Cryptosporidium* detected in pigs are *C. suis* and *C. scruforum; C. suis* is prevalent in pigs worldwide but causes few clinical signs (Enemark et al., 2003). *Cryptosporidium scruforum* is a fairly new species, only described in 2013 (Kvac et al., 2013), again, this species does not seem to cause any clinical disease in pigs yet is prevalent in adult pigs worldwide. It does not appear infective to pigs under 8 weeks of age (Kvac et al., 2014).

1.3.2 Species of *Cryptosporidium* in poultry

Three species of *Cryptosporidium* are currently known to infect birds; *C. meleagridis, C. baileyi* and *C. galli*. Avian cryptosporidiosis was first described in 1929 but was not formally recognised until 1955 when *C. meleagridis* was reported in turkeys (Slavin, 1955), *C. meleagridis* is found in the small and large intestine and bursa of infected birds. *Cryptosporidium meleagridis* has been reported in many different species of birds and also in humans (Ng-Hublin et al., 2013; Silverlas et al., 2012; Wang et al., 2014b). The second species of *Cryptosporidium* to be reported in birds was *C. baileyi* in chickens in 1986 (Current et al., 1986), this species is more commonly associated with respiratory cryptosporidiosis in broiler chickens and is the most common species of avian *Cryptosporidium*. Also described from chickens is *C. galli* which was discovered in 1999 but was redescribed in 2003 (Ryan et al., 2003a). Like *C. meleagridis* this species is also found in the small intestine, large intestine and in the epithelium of the proventriculus and is associated with clinical disease and mortality (Blagburn et al., 1991; Tumova et al., 2002). The three avian adapted species are morphologically different and can be distinguished by morphological difference in the size and shape of the oocysts.

1.3.3 Species of *Cryptosporidium* in non-mammalian vertebrates

1.3.3.1 Fish

In fish three species have been reported but it is possible that there are more which remain undiscovered as studies into the species of *Cryptosporidium* found in fish are scarce. The first species in fish to be described was *C. molnari* infecting the stomach of gilthead sea bream and European sea bass with few clinical signs (Alvarez-Pellitero and Sitja-Bobadilla, 2002). The other reported species of *Cryptosporidium* infecting fish is *C. scophthalmi* which is found in the intestinal epithelium of turbot. So far it appears that juvenile fish are most frequently infected with *Cryptosporidium* while it has rarely been detected in adults (Alvarez-Pellitero et al., 2004). Infected fish tend to show no signs of clinical infection but do show intestinal distension at necropsy. A more recent study, has also detected *C. parvum* in fish from Papua New Guinea (Koinari et al., 2013) as well as a novel *Cryptosporidium* species which is also zoonotic. The

most recently described species of *Cryptosporidium* is a species identified in the stomach of a guppy (Ryan et al., 2015).

1.3.3.2 Amphibians and reptiles

Four species of *Cryptosporidium* have been reported in amphibians and reptiles, *C. fragile, C. varanii, C. serpentis* and *C. ducismarci*. As its name suggests, *C. fragile* is the most delicate species of *Cryptosporidium* and was first described in the stomach of the black-spined toad (Jirku et al., 2008) and was named due to its fragile nature. Unlike other species of *Cryptosporidium, C. fragile* will crumple on contact with hypertonic solutions and disintegrate after just 4 weeks in water (Jirku et al., 2008). *Cryptosporidium* has been reported in many species of snakes and other reptiles, the first to be identified was *C. serpentis* which is a gastric species and was found in four snake species in 1977 (Brownstein et al., 1977). It is believed that *C. serpentis* may actually be a name which covers many other species of *Cryptosporidium* infecting snakes which have not yet been described (Fayer, 2010) as morphological data from many different species of reptiles indicates the possibility of multiple unique groups (Upton et al., 1989; Xiao and Ryan, 2004; Xiao et al., 2004). *Cryptosporidium varanii* was first described in an Emerald Monitor lizard (Pavlasek and Ryan, 2008b); it has since been identified in many other lizard and snake species. The most recently discovered *Cryptosporidium* species in a reptile is *C. ducismarci* which was described from tortoises (Traversa, 2010) and which caused fatal intestinal disease in two different species of tortoise. As with fish it is possible that many more species of *Cryptosporidium* infecting reptiles and amphibians may exist but not enough studies have been carried out to confirm this.

1.3.4 Species of *Cryptosporidium* in humans

In humans there are two species which are routinely diagnosed in clinical cases of cryptosporidiosis; these are the zoonotic species *C. parvum* and the human adapted species *C. hominis* (Morgan-Ryan et al., 2002). These species account for over 90% of human infections worldwide (Chalmers and Giles, 2010) and 96% of clinical cases in the UK are attributed to these two species (Hadfield et al., 2011). *Cryptosporidium parvum* infections peak in the spring months while *C. hominis* is more common in the autumn months (Chalmers et al., 2009a), this is

thought to be related to springtime calving and lambing and an increase in people participating in outdoor activities at this time of year. Other species which commonly infect humans include; *C. meleagridis* (Chalmers and Giles, 2010; Xiao and Fayer, 2008) in the UK and *C. felis*, *C. canis* and *C. meleagridis* in other parts of the world (Cama et al., 2008). Sporadic infections with other *Cryptosporidium* species have also occurred although these are not always associated with disease and may just be asymptomatic infections (Helmy et al., 2013; Robinson et al., 2008; Waldron et al., 2010). While most outbreaks in the UK are associated with either *C. hominis* or *C. parvum* one outbreak in 2008 was caused by *C. cuniculus* (Chalmers et al., 2009b; Robinson et al., 2010). *Cryptosporidium cuniculus* was originally reported as causing infection in rabbits in 1979 (Inman and Takeuchi, 1979) and was believed at this time to be host specific. Following the human outbreak of 2008 it was discovered that *C. cuniculus* is infective to humans and rabbits and under experimental conditions immunosupressed gerbils and mice can become infected (Robinson et al., 2010). There are no clinical signs recorded for rabbits. This species is very similar to *C. hominis* at several loci including 18S SSU, heat shock protein 70 (HSP), actin, LIB13 and *Cryptosporidium* oocyst wall protein (COWP), in 2010 a redescription of *C. cuniculus* was published (Robinson et al., 2010). In 2012 a new human adapted species was recognised and named *C. viatorum,* this species is associated with travellers who have visited the Indian subcontinent (Elwin et al., 2012b).

1.3.5 Other *Cryptosporidium* species

1.3.5.1 Companion animals

In the UK other *Cryptosporidium* species are commonly found in domestic pets, in one study it was found that 20% of cats and dogs were shedding *C. felis* and *C. canis* respectively (Mtambo et al., 1991). Worldwide the prevalence of *Cryptosporidium* in these companion animals ranges from 0-29.4% in cats and 0.5-44.1% in dogs (Lucio-Forster et al., 2010), *C. parvum* has been detected in dogs previously but these animals are not thought to be an important factor in zoonotic transmission of the parasite (Giangaspero et al., 2006; Lucio-Forster et al., 2010).

1.3.5.2 Horses

Cryptosporidium was first reported in horses in the late 1970s in immunodeficient foals (Snyder et al., 1978). At this time it was generally believed that in animals, as well as humans, cryptosporidiosis was only a problem where the host was immunocompromised in some way. Since the initial report of *Cryptosporidium* in foals there have been many reports of the parasite in immunocompetent horses (Santin, 2013). The most common clinical sign of cryptosporidiosis in foals is diarrhoea (Grinberg et al., 2009), in adult horses most infections tend to be asymptomatic (Majewska et al., 2004; Sturdee et al., 2003) although diarrhoea in adult horses has been reported (McKenzie and Diffay, 2000). The most commonly detected species in horses is *C. parvum* (Chalmers and Grinberg, 2005; Cole et al., 1998; Perrucci et al., 2011).

1.3.5.3 Wildlife

In British wildlife (rabbits, rodents, foxes and deer) various species and genotypes of *Cryptosporidium* have been identified although relatively few studies into wildlife hosts in the UK have been carried out (Appelbee et al., 2005). One study which examined a large number of wildlife hosts as well as livestock and horses identified *Cryptosporidium* oocysts, believed at the time to be *C. parvum,* in ~30% of faeces from small wild mammals (Sturdee et al., 2003) and in another study, oocysts were identified in 63% of brown rat faecal samples (Webster and Macdonald, 1995). A study in the UK investigating *Cryptosporidium* species in a water catchment found *C. ubiquitum* in deer samples and in foxes *C. bovis* and *C. parvum* were identified (Robinson et al., 2011). Although this study found no evidence of *Cryptosporidium* in rabbits an outbreak in humans in 2008 was linked to the rabbit genotype, now identified as a separate species *C. cuniculus* (Chalmers et al., 2009b; Robinson et al., 2010). A recent study investigating *Cryptosporidium* species and genotypes found in livestock and deer in a water catchment in north east Scotland reported a prevalence of 69.23% in deer (80% in red deer and 33% in roe deer) the majority of the samples were identified as *C. parvum* with the remainder identified as the *Cryptosporidium-deer* genotype (Wells et al., 2015).

In other parts of the world different species have been identified in other wildlife hosts; *C.wrairi* was first identified in guinea pigs in 1966, this species has only ever been identified from guinea pigs and appears to be host specific (Jervis et al., 1966; Vetterling et al., 1971). In marsupials two species have been reported, *C. fayeri* which was isolated from a red kangaroo (Ryan et al., 2008) and *C. macropodum* which was first reported in an eastern grey kangaroo (Power and Ryan, 2008) and has since been reported in other marsupials such as; swamp wallabies, western grey kangaroos and red kangaroos (Power, 2010). Neither *C. fayeri* nor *C. macropodum* have been associated with clinical disease in marsupials (Yang et al., 2011).

1.4 Diagnosis and identification of species

Differentiation of *Cryptosporidium* species found in both humans and farm livestock is important as it enables disease epidemiology involving different species to be understood and in human disease cases outbreaks may be traced back to the original source and help inform control strategies (Chalmers et al., 2009a; Gormley et al., 2011). Many studies have examined the epidemiology of *Cryptosporidium* in humans (Caccio, 2005; Chalmers et al., 2009a; Fayer et al., 2000a; Xiao, 2010). However, similar studies in farm livestock are few and often only focus on the neonatal age group. Longitudinal studies in livestock are not very common though some have been carried out in France, the USA, the UK, Australia and Scandinavia (Brook et al., 2009; Follet et al., 2011; Robertson et al., 2010; Santin et al., 2008; Sturdee et al., 2003; Yang et al., 2014). During human outbreaks of cryptosporidiosis it is usual for the parasite to be speciated and even genotyped whereas in veterinary diagnosis often only the presence of the parasite is confirmed. The most common methods for detection of *Cryptosporidium* in the UK are; microscopy, enzyme immunoassays (EIA) and polymerase chain reaction (PCR) (Chalmers et al., 2010).

1.4.1 Microscopic Identification

Identifying only the presence of the parasite by microscopy can be problematic for diagnosis as many *Cryptosporidium* species do not cause disease in some hosts, for example even under experimental conditions *C. ryanae* and *C. bovis* do not cause diarrhoea in calves (Fayer et al., 2008; Fayer et al., 2005). There

are few morphological differences between *Cryptosporidium* species and internal structures cannot be easily seen and it is not possible to speciate most *Cryptosporidium* species by microscopy alone. There are some exceptions to this rule with some species of *Cryptosporidium* such as *C. andersoni* and *C. muris* being larger in size than the others (Lindsay et al., 2000). Many species of *Cryptosporidium* are not host specific and multiple species can often be found in the same host. The oocysts of *Cryptosporidium* are very small (3-8 µm) and between species there is overlap in oocyst size. Under microscopic examination *Cryptosporidium* oocysts can be difficult to distinguish from other small particles such as yeasts, moulds and faecal debris. Various techniques for detection of oocysts by microscopy exist (Figure 1-2). The most common method for detection of *Cryptosporidium* by microscopy in the UK is auramine phenol staining. With this method the oocysts are stained a bright greenish yellow against a dark background and sensitivity and specificity of this staining method is good (~92% sensitivity and 100% specificity). The 2nd most common method used in UK diagnostic laboratories for human specimens is modified Ziehl-Neelsen (mZN). Using mZN the oocysts appear red on a blue/green background and the sensitivity of this method is ~75% with 100% specificity, this technique is also commonly applied in veterinary diagnostics as well. Finally another common method of detection by microscopy is 4,6' diamidino-2-phenylindole dihydrochloride (DAPI) staining which stains the nuclei of viable oocysts and they appear blue under UV light, oocysts can also be detected without staining, using phase contrast microscopy, (Chalmers et al., 2011)

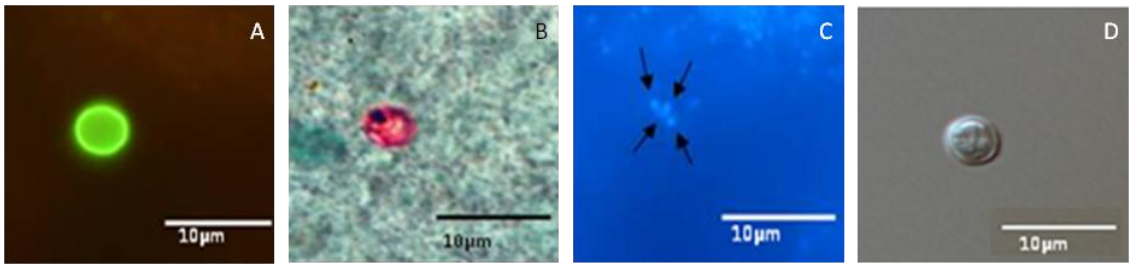


Figure 1-2: *Cryptosporidium* oocysts as seen through a microscope using different staining methods.
Phenol Auramine (AP) staining (A) stains the oocysts bright green, Modified Ziehl-Neelsen (mZN) (B) stains oocysts pink against a blue/green background, DAPI stains the nuclei of viable oocysts making them appear blue (C) and it is possible to visualize oocysts using phase-contrast microscopy without any staining (D). Images from (Robinson et al., 2010).

Microscopy techniques are commonly used for diagnostic purposes but most often in a research setting molecular tools are used instead as these are much more sensitive and provide far more additional information (Chalmers and Katzer, 2013). Many conserved genes have been targeted for the purpose of species differentiation including; those encoding 18S rRNA (Xiao et al., 1999), 70 kDa heat shock protein (HSP70) (Sulaiman et al., 2000), *Cryptosporidium* oocyst wall protein (COWP) (Xiao et al., 2000b) and the actin gene (Kim et al., 1992). Probably the most commonly used of these is the 18S rRNA gene as in addition to containing regions that vary between species there are several regions which are conserved within the *Cryptosporidium* genus. In each *Cryptosporidium* oocyst there are 20 copies of this gene (Le Blancq et al., 1997; Morgan and Thompson, 1999) meaning that sensitivity is increased when compared with a single-copy gene.

1.4.1 Enzyme Immunoassays

Enzyme immunoassays (EIA) are very common in the UK for detection of *Cryptosporidium* in human faecal samples and are very sensitive in detecting *C. parvum* (Garcia et al., 1987) but the ability of these kits to detect other *Cryptosporidium* species has not been thoroughly assessed. Some studies show a reduction in sensitivity when other *Cryptosporidium* species (*C. andersoni*) are present (Lindergard et al., 2001). EIAs use rabbit anti-*Cryptosporidium* polyclonal antibodies to capture the *Cryptosporidium* antigen from the stool supernatant. A second set of goat anti-*Cryptosporidium* polyclonal antibodies are then added, which sandwiches the captured *Cryptosporidium* antigen (Ungar, 1990). Sometimes these assays can be combined to allow the detection of other parasites such as *Giardia* at the same time. EIA are very sensitive (100%) and specific (~91-93%) but can be expensive to perform (Chalmers et al., 2011) which may prohibit their use in large studies.

1.4.2 Molecular detection of *Cryptosporidium*

Species differentiation using molecular methods can be done in a few different ways; these begin with DNA extraction from oocysts and PCR amplification of the gene(s) of interest. DNA extracted from oocysts can be amplified using standard or nested PCR methods. In standard PCR one pair of forward and reverse primers

are used to amplify a gene or region of a gene, whereas in nested PCR two sets of primers are used. The first pair (external) amplify the gene of interest and in a second round; internal primers amplify a shorter sequence of the amplicon produced in the first PCR. This is useful if the sample only contains a small amount of DNA as it results in more DNA copies than standard PCR. The PCR products are separated by electrophoresis on an agarose gel and visualised by staining with a stain such as ethidium bromide which binds to nucleic acids and can be viewed under UV light. Species can be differentiated using restriction enzymes to digest PCR products into fragments of different sizes (PCR-Restriction Fragment Length Polymorphism (RFLP)). These fragments can be visualised on the agarose gel, producing varying banding patterns depending on species (Spano et al., 1997; Sulaiman et al., 1999). Another commonly used method is DNA sequencing of the PCR products, the PCR product is purified and re-amplified using either the same primers in the initial PCR or primers specifically designed for sequencing and fluorescently labelled nucleotides. The labelled nucleotides emit light at specific wavelengths giving the sequence of the DNA fragment which can be analysed using various computer based programs.

Another PCR method which can be used to detect and speciate *Cryptosporidium* parasites is real-time PCR which is considered to be the "gold-standard" for *Cryptosporidium* detection (Chalmers et al., 2011) as this method is the most sensitive and can detect as few as 2 oocysts per PCR (Hadfield et al., 2011). In real-time PCR the amplification of the DNA can be tracked in "real time" and the amount of DNA present can be quantified, this is an advantage over standard PCR which can only indicate the presence of parasite DNA but cannot quantify the amount.

1.5 *Cryptosporidium* subtyping

As well as speciating *Cryptosporidium* positive samples it can be useful to further subtype particular species, which can be informative in epidemiological studies and disease outbreaks to identify origin or source of particular subtypes and to study population genetics (Gatei et al., 2008; Mallon et al., 2003; Morrison et al., 2008; Wang et al., 2014b). Subtyping can be carried out by multilocus sequence typing (MLST) using several microsatellites to identify

alleles, assigning a parasite a multilocus genotype (MLG). MLST is useful for studying population genetics but it is not always necessary to examine the parasite in such detail. Many studies have subtyped certain *Cryptosporidium* species (*C. parvum, C. hominis, C. meleagridis, C. ubiquitum* and *C. cuniculus*) using the 60-kDa glycoprotein gene (*gp60*). This is informative enough to show inter-species variation and has also been linked to differences in clinical disease (Adamu et al., 2014; Cama et al., 2008) and, in some cases host specificity (Wang et al., 2014a). For example, some *gp60* subtype families (IIc, IIb, IIe-IIl) of *C. parvum* have only been detected in humans but never in animal hosts. This is expanded on in section1.5.2.

1.5.1 *gp60* Subtyping

The GP60 protein is expressed on the surface of invading stages of the parasite and is a target for neutralising antibodies (O'Connor et al., 2007). It is formed from two cell surface glycoproteins *gp15* and *gp45*; *gp45* is also referred to as *gp40* and *S45* (Strong et al., 2000; Strong and Nelson, 2000; Winter et al., 2000) and *gp15* as *cp17* and *S16* (Winter et al., 2000). The *gp60* gene is similar to a microsatellite as it contains multiple repeats of three tri-nucleotides (TCA, TCG and TCT) at the 5' end of the gene.

Sequence analysis of the *gp60* gene is commonly used for genotyping *Cryptosporidium* species and often *gp15* is used as a microsatellite marker for multilocus genotyping to identify *Cryptosporidium* subtypes (Strong et al., 2000). In addition to variation in the number of tri-nucleotide repeats in the microsatellite region of gp*60* there are also extensive sequence differences in non-repeat regions of the gene. These differences enable us to define *Cryptosporidium* species into subtype families (Sulaiman et al., 2005); the name of *gp60* subtypes always starts with identification of the subtype family e.g. IIa (this is the most commonly recognised *C. parvum* subtype family in the UK) followed by the number of TCA (A), TCG (G) and TCT (T) repeats within the microsatellite region (Sulaiman et al., 2005). In some subtypes of *C. parvum,* either within or immediately after the microsatellite region there is a six base-pair repeat ACATCA which is counted and represented by "R", in addition, some *C. hominis* subtypes have a 13 or 15 base-pair repeat (5' –AAGACGGTGAAGG3'/ 5' –AGGACGGTGGTAAGG-3') at the end of the microsatellite region also

represented by “R”. The most commonly identified *C. parvum gp60* subtype found in the UK is IIaA15G2R1 which means that the parasite belongs to *C. parvum* subtype family IIa and contains 15 copies of the trinucleotide repeat TCA, two copies of TCG, no copies of TCT and one copy of the 6 base-pair sequence ACATCA (Figure 1-3).

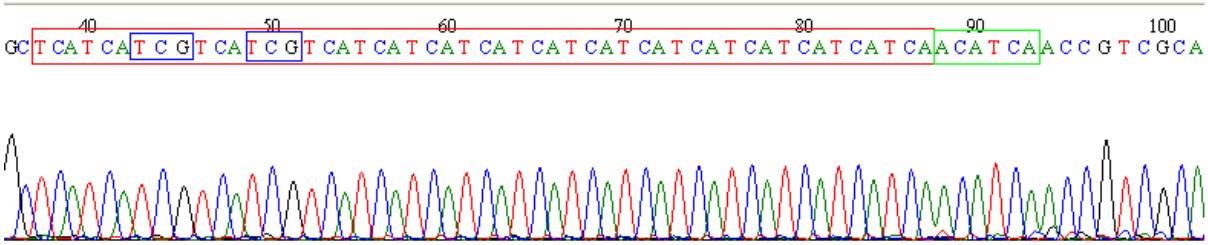


Figure 1-3: *gp60* sequence showing microsatellite region with TCA, TCG and ACATCA repeats highlighted.

The main advantage to using *gp60* as a single locus marker is that extensive use of this gene worldwide means comparisons of subtypes can easily be made between studies, countries and host species. It is also possible to link *gp60* subtypes with biological characteristics and clinical disease (Xiao, 2010) as, unlike many of the other genes which are used for subtyping, it is a functional protein which is involved in host cell attachment and parasite invasion (O'Connor et al., 2007). One disadvantage of using this single locus is that, at present, it can only be used to subtype certain species (*C. parvum, C. hominis, C. meleagridis, C. ubiquitum* and *C. cuniculus*).

1.5.2 *C. parvum gp*60 Subtype Diversity

In many countries high subtype diversity of *C. parvum* has been reported from both human and ruminant studies. In Northern Ireland a study which analysed the gp*60* sequence of 214 neonatal calves found 15 different subtypes all from the IIa subtype family (Thompson et al., 2007) and in the Netherlands 160 *C. parvum* positive bovine samples were subtyped by *gp60* sequence analysis and 17 subtypes of IIa and III subtype families were identified (Wielinga et al., 2008).

Subtyping of *C. parvum* and *C. hominis* with *gp60* has been used worldwide, it has been shown that some *C. parvum* subtype families (IIa and IId) are found in

both humans and ruminants, whereas other *C. parvum* subtypes (IIc, IIb, IIe-IIl) have only been identified in humans (Alves et al., 2003; Cama et al., 2008; Hijjawi et al., 2010). Even within ruminants it has been found that some *C. parvum* subtypes preferentially infect one ruminant species over another. In areas of Spain where both IIa and IId are found IIa predominantly infects calves while IId predominantly infects lambs and goat kids (Quilez et al., 2008a; Quilez et al., 2008b). The most common subtype of *C. parvum* is IIaA15G2R1 which has a worldwide distribution and is often found in both humans and ruminants (Broglia et al., 2008). Within *C. hominis* subtypes significant variations in clinical disease and virulence have also been found in certain areas of Peru and Ethiopia in patients suffering from HIV (Adamu et al., 2014; Cama et al., 2008; Cama et al., 2007). It has been previously suggested that differences in clinical manifestations may be due to the immune status of the infected person (Flanigan, 1994) but the results from these studies suggest that there are differences in the parasite in addition to host immune factors. Between species, *C. parvum* was likely to be the most pathogenic as it was significantly associated with chronic diarrhoea and vomiting while *C. meleagridis* was less pathogenic. Within species, those infected with *C. hominis* subtype family Id were most likely to suffer from diarrhoea while those infected with subtype family Ia had fewer clinical signs. It is also possible that some people have a genetic predisposition that makes them more susceptible to infection with *Cryptosporidium*. Fayer et al (2000) showed that children with B*15 HLA I allele and the DQB1* 0301 HLA II allele were more likely to suffer from cryptosporidiosis than those with a different allele.

Although most studies subtyping *Cryptosporidium* species are studies examining human samples or outbreaks there are more and more studies being carried out to investigate the subtypes of *Cryptosporidium* infecting farm livestock. One cross-sectional study of neonatal calves from 41 farms in the South of England demonstrated that most farms have a single predominant *gp60* subtype (Brook et al., 2009). The findings in this study tie in with other studies which show that on individual farms *C. parvum gp60* subtypes seem stable but that there is wide genetic variation between farms. A study carried out in Scotland on the population genetics of *Cryptosporidium* species by microsatellite analysis of isolates from human, cattle and sheep sources showed that genetic diversity in

bovine isolates can be linked with cattle movement (Morrison et al., 2008). Some other studies which have looked at the *C. parvum gp60* subtypes of calves have found similar results to this but another study in the UK found that IIaA15G2R1 was not the most commonly detected subtype. The study examined 779 faecal samples collected from neonatal diarrhoeic calves from farms across Northern Ireland. All of the *C. parvum* positive samples (n=213), were subtyped at the *gp60* locus, in total 16 different subtypes were identified all from allele family IIa. The most prevalent subtype in this study was IIaA18G3R1 (n=120) (Thompson et al., 2007) which has also been identified in humans from Northern Ireland (Zintl et al., 2009).

1.6 Transmission of *Cryptosporidium*

Cryptosporidium oocysts are transmitted between hosts via the faecal-oral route, either directly from contact with faeces of infected animals or indirectly through environmental contamination or from ingestion of contaminated food or water.

There are some reports of veterinary students, typically from urban areas, becoming infected with *C. parvum* when they start working with farm livestock (usually calves) during their studies (Gait et al., 2008; Pohjola et al., 1986; Preiser et al., 2003) as well as outbreaks amongst members of the public associated with petting zoos or farm visits (Pritchard and Fleetwood, 1995) (Gormley et al., 2011). Drinking water outbreaks can be very serious as many people can become infected in a short space of time. The oocysts of *Cryptosporidium* are very small and as such it can be difficult to eliminate them from municipal supplies as the filters used are larger than the oocysts themselves. It is also not possible to chemically inactive the oocysts (Pollock et al., 2008) by chlorination or other methods commonly used to treat drinking water. More recently in some areas the use of ozone and UV light have been very useful in inactivation of *Cryptosporidium* oocysts in drinking water (Cho and Yoon, 2007). The world's largest waterborne *Cryptosporidium* outbreak occurred in Milwaukee in 1993. During this outbreak ~400,000 people were affected. The source of the outbreak was never confirmed at the time but run-off from cattle pastures due to ice melt was one suggestion (Mac Kenzie et al., 1994). Retrospective analysis of samples collected at the time of the outbreak has

shown that patients were in fact infected with *C. hominis* rather than *C. parvum*. This indicates that the source of the infection was anthroponotic rather than zoonotic . Foodborne transmission of *Cryptosporidium* can occur when food items, such as vegetables and salad items are washed with contaminated water during processing, from contamination of food by a food handler or contact with infected animal faeces. Foodborne transmission of cryptosporidiosis seems to be much lower than waterborne transmission, this is either because there is less foodborne transmission or because of under reporting of outbreaks (Robertson and Chalmers, 2013).

Anthroponotic transmission of *C. hominis* has been associated with those working in caring environments such as nursery and care workers who may have close contact with faeces from infected individuals (Joachim, 2004). There have also been waterborne outbreaks associated with human to human transmission via drinking water (Mason et al., 2010; Widerstrom et al., 2014) and recreational waters (Coetzee et al., 2008).

1.6.1 Infective Dose

Attempts have been made to quantify the minimum infective dose of *Cryptosporidium* oocysts of different species in different host species. The results vary between study even when the same *Cryptosporidium* and host species is being examined. One study carried out on human volunteers with *C. parvum* reported an infective dose of 132 oocysts (DuPont et al., 1995). As has been previously discussed variation in the outcome of clinical disease occurs depending on the isolate used, another paper reported differences in the infective dose among *C. parvum* isolates in human volunteers. The infective dose for *C. parvum* IOWA isolate was 87 oocysts, 1042 oocysts for *C. parvum* UCP isolate and only 9 oocysts for *C. parvum* TAMU isolate (Okhuysen et al., 1999). One study in calves (infected with *C. parvum*) found that the infective dose for oocyst shedding only was 5.8 oocysts, scour only was 9.7 oocysts and for oocyst shedding with diarrhoea was 16.6 oocysts (Zambriski et al., 2013b). The infective dose is thought to be very low due to the rapid life cycle and ability of the parasite to auto-infect host cells.

1.6.2 Oocyst Shedding (calves)

Infected hosts can shed huge numbers of oocysts per day, which are immediately infective to other susceptible hosts (Nydam et al., 2001; Zambriski et al., 2013a) meaning that infection can pass very quickly between animals kept in close contact to one another. Infected calves will begin to shed infective oocysts 4-9 days after initial infection (depending on infective dose) and can shed large numbers of oocysts each day (Faubert and Litvinsky, 2000). Studies have reported oocyst shedding in calf faeces to be around 10^4 - 10^8 oocysts per gram of faeces (Fayer et al., 1998), the number of oocyst shed in the faeces depends on the initial infective dose (Fayer et al., 2010a; Rieux et al., 2013; Zambriski et al., 2013a). Excretion of oocysts normally lasts for 6-14 days (Fayer et al., 1998; Nydam et al., 2001; Zambriski et al., 2013a) and high oocyst excretion is more usually associated with calves younger than one month (O'Handley et al., 1999; Ongerth and Stibbs, 1989). Animals infected with a higher dose will not shed any longer than those receiving a lower dose but they will shed more oocysts throughout the duration of shedding (Zambriski et al., 2013a).

1.7 Cryptosporidiosis

Not all species of *Cryptosporidium* cause similar disease manifestations. In most mammals' infection with *C. parvum* causes self-limiting watery diarrhoea, loss of appetite and abdominal pain (Clark and Sears, 1996; Klein et al., 2008; Tzipori et al., 1983; Tzipori and Ward, 2002). In other host species different *Cryptosporidium* species will cause similar clinical disease such as; *C. hominis* in humans, *C. meleagridis* and *C. galli* in birds, usually chickens; *C. serpentis* in snakes; *C. varanii* in lizards and *C. xiaoi* in goats whereas other *Cryptosporidium* species manifest themselves in other ways. For example, in birds the most common form of cryptosporidiosis is respiratory cryptosporidiosis which is caused by *C. baileyi* (Lindsay et al., 1986). Other species of *Cryptosporidium* appear to be non-pathogenic in some hosts such as *C. ryanae* and *C. bovis,* these are commonly found in calves but experimental infections have shown that although infected animals shed oocysts infection with these species is not associated with clinical disease (Fayer et al., 2008; Fayer et al., 2005). In some cases and in immunosupressed individuals disease caused by *C. parvum* can be severe, lasting

for several weeks, spreading to other areas of the body, as opposed to the intestines, such as the respiratory tract causing respiratory disease and may even lead to death (Denkinger et al., 2008; Hojlyng and Jensen, 1988; Moore and Frenkel, 1991; Mor et al., 2010).

Diarrhoea caused by *C. parvum* can occur in two ways; malabsorptive diarrhoea is caused by loss of enterocytes and blunting of villi (Figure 1-4) which reduces the intestinal surface leading to decreased water and nutrient absorption (Klein et al., 2008; Tzipori and Ward, 2002). Secretory diarrhoea occurs when intestinal secretion is increased. Some infectious organisms produce toxins which stimulate intestinal secretion by invading host epithelial cells (Clark and Sears, 1996) and there have been reports of patients infected with *Cryptosporidium* suffering from profuse watery diarrhoea, which is very similar to the secretory diarrhoea that is caused by the cholera (*Vibrio cholera*) toxin. A study in 1994 identified enterotoxin-like activity in stool supernatant from *C. parvum*-infected calves (Guarino et al., 1994) and others believe that *C. parvum* might simulate proinflammatory cytokines. Intestinal damage caused by infection can lead to a slight reduction in growth rates but the intestinal absorption is generally restored three weeks after infection and no long-term damage to the gut occurs (Klein et al., 2008).

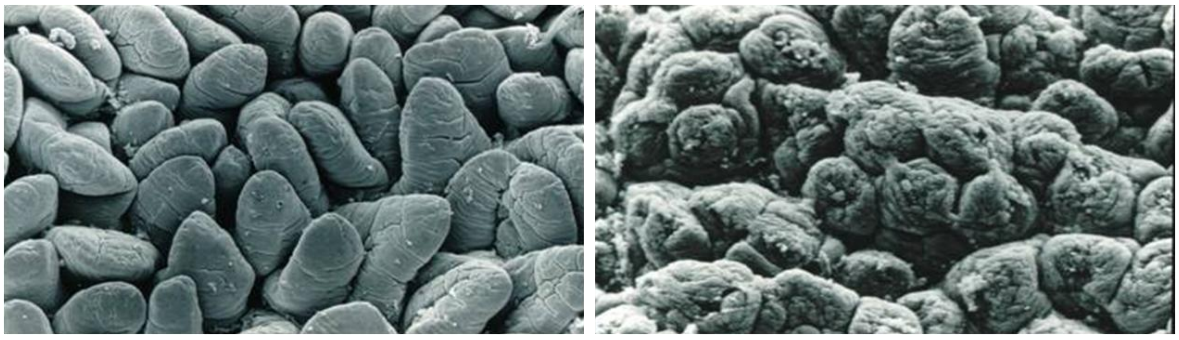


Figure 1-4: Healthy gut villi (left) and damaged gut caused by *C. parvum* infection (right) in a calf.

1.7.1 Disease in farm livestock

Cryptosporidiosis in farm livestock usually lasts around two weeks in otherwise healthy animals and has no long-term implications on animal health except in some severe cases when lambs and chickens may suffer from a reduction in

growth (de Graaf et al., 1999; Goodwin et al., 1996; Sweeny et al., 2011a). The economic losses associated with *Cryptosporidium* have never been calculated but losses would include cost of treatment of enteritis, reduced feed conversion and production efficiency and, of course, losses due to animal death.

The majority of animals infected with *Cryptosporidium* survive but in some host species animals which have been infected may have lower growth rates than those that have not, lambs infected with *C. parvum* were shown to have reduced growth rate, live-weight and carcass quality than those not infected (Sweeny et al., 2011b).

Despite most reports stating that cryptosporidiosis is generally self-limiting with no long-term health implications there are several anecdotal incidences of very severe cryptosporidiosis leading to high morbidity and mortality within herds and flocks with some farmers having no choice but to leave the industry due to lack of profitability.

1.7.1.1 Cattle

Cryptosporidiosis was first reported in cattle in the early 1970's (Meuten et al., 1974; Panciera et al., 1971) but the diarrhoea caused in these animals was also associated with other viral and bacterial infections and could not be solely attributed to *Cryptosporidium*. In 1983 neonatal diarrhoea in calves was reported with *Cryptosporidium* species as the sole infective agent (Tzipori et al., 1983) and it is now recognised that the disease is endemic worldwide. Infection with *C. parvum* in cattle tends to be located in the small intestine at the ileocaecal junction but the parasite has also been found in the caecum, colon and duodenum (Pohlenz et al., 1978; Sanford and Josephson, 1982). Different species of *Cryptosporidium* are found in different locations. In adults infected with *C. andersoni* the parasite is found in the abomasum (Lindsay et al., 2000). Death of young calves caused by *Cryptosporidium* is usually attributed to dehydration due to severe diarrhoea (Sanford and Josephson, 1982).

Different age groups of cattle are commonly infected with distinct species of *Cryptosporidium* (Figure 1-5) although there are sometimes exceptions to this. Clinical disease caused by *Cryptosporidium* is usually limited to neonatal

livestock with older animals (>6 weeks) exhibiting only asymptomatic shedding of oocysts (Fayer et al., 2006) and is caused by *C. parvum*; whereas the species that infect older calves (*C. bovis* and *C. ryanae*) have not been associated with any clinical disease (de Graaf et al., 1999; Fayer et al., 2008; Fayer et al., 2005). *Cryptosporidium andersoni* tends to affect the abomasum of adult cattle but this species does not cause diarrhoea and much fewer oocysts are shed compared with *C. parvum* infection (Kvac and Vitovec, 2003), infection with this species tends to cause mild clinical disease and affects weight gain and milk production (Anderson, 1987; Esteban and Anderson, 1995; Ralston et al., 2010).

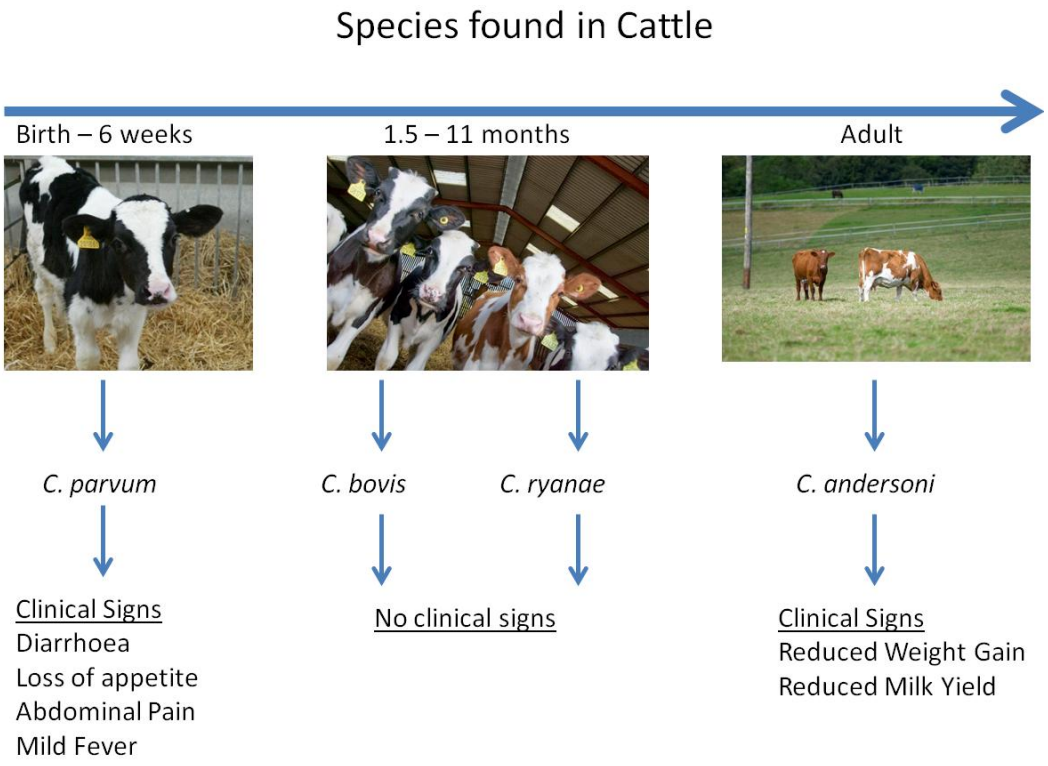


Figure 1-5: Species of *Cryptosporidium* generally associated with different age groups of cattle.
It is commonly accepted that different species of cattle-adapted *Cryptosporidium* species are found in different age groups of cattle. *C. parvum* is commonly found in young calves, *C. bovis* and *C. ryanae* in older calves and *C. andersoni* in adult cattle (Santin et al., 2004).

1.7.1.2 Sheep and goats

In sheep, cryptosporidiosis was first reported in lambs less than three weeks old suffering from diarrhoea on a farm in Australia (Barker and Carbonell, 1974). As with cattle, *Cryptosporidium* is now recognised as one of the main etiological agents of lamb diarrhoea and in lambs is associated with high morbidity and

mortality in infected flocks (de Graaf et al., 1999; Sari et al., 2009). Cryptosporidiosis in lambs appears to cause more mortality than in calves, this is perhaps because lambs can become dehydrated much more quickly than calves. Australia was also the first place where cryptosporidiosis of goats was reported (Mason et al., 1981), it is now considered a very important disease of goats due to the high morbidity (up to 100%) and mortality (up to 50%) in some goat herds (Johnson et al., 1999; Paraud et al., 2010; Vieira et al., 1997). In small ruminants the infection causes depression, anorexia, abdominal pain and diarrhoea. The diarrhoea is usually accompanied by the shedding of large numbers of oocysts, usually lasting three to five days but in severe cases up to two weeks. In small ruminants such as sheep and goats infection normally occurs in the jejunum and ileum but can spread to other areas in the small and large intestine (Snodgrass et al., 1984).

1.7.1.3 Poultry

In domestic poultry the most common form of cryptosporidiosis is respiratory rather than diarrhoeic except in turkeys. Birds are also known to suffer from renal and ocular cryptosporidiosis.

Cryptosporidium meleagridis infects the intestinal tract, bursa of fabricius and cloaca (Ryan, 2010) in turkeys and chickens but clinical disease seems to affect only turkeys (Slavin, 1995), in other birds asymptomatic infections have been reported (Abe and Iseki, 2004). *C. baileyi*, in addition to infection of the intestinal tract, bursa of fabricius and cloaca also infects the respiratory and urinary tract in turkeys, chickens and ducks (Lindsay et al., 1986). It is the most common cause of cryptosporidiosis in poultry, especially chickens where it normally manifests itself as respiratory disease with only occasional intestinal disease. *C. baileyi* infection has also been associated with a lower body weight in broiler chickens (Goodwin, 1996). The third avian-adapted species, *C. galli*, does not develop in either the intestines or respiratory tract but instead develops in the proventriculus, causing diarrhoea; this species has been associated with high mortality in birds (Ryan et al., 2003a).

1.7.2 Humans

At present it is known that humans can be infected with ~12 of the currently recognised species (*C. hominis, C. parvum, C. viatorum, C. muris, C. felis, C.suis, C. canis, C. meleagridis, C. ubiquitum, C. fayeri, C. bovis* and *C. cuniculus*) though some of these infections may have been transient infections and infections with some species have only been reported once (Elwin et al., 2012a). Humans are most commonly infected with *C. hominis* and *C. parvum* (Hadfield et al., 2011) and it was originally thought that *C. hominis* infected only humans; however, there are some reports of *C. hominis* in other animals; a rhesus monkey (Ye et al., 2012); a dugong (Morgan et al., 2000); Canadian geese (Zhou et al., 2004); a sheep from Australia (Ryan et al., 2005); two cattle from Scotland (Smith et al., 2005) and a pygmy goat and a Texel lamb in the UK (Giles et al., 2009).

Cryptosporidiosis in humans is prevalent worldwide in both developed and developing countries. Children and the elderly are more susceptible to disease than young adults. Children tend to acquire the infection shortly after, or during weaning (Tzipori, 2002). Infection of immunocompetent people with *Cryptosporidium* tends to cause self-limiting diarrhoea, however, a recent report showed that *Cryptosporidium* is the second biggest cause of infant diarrhoea and death in Africa and Asia (Striepen, 2013) which demonstrates that cryptosporidiosis is not always self-limiting and mild. Time from infection to clinical signs can vary depending on host variables such as age and any previous exposure and also on the parasite (age of oocysts, species/genotype); *C. hominis* tends to cause more severe and extra-intestinal disease than other species (Cama et al., 2007). Infection generally occurs in the small intestine but can spread through the large intestine and into the stomach. Infections concentrated in the small intestine of humans have a tendency to be more severe and result in more watery diarrhoea than infections in the distal ileum or bowel, which are often asymptomatic. Where diarrhoea lasts more than seven days serious dehydration and weight-loss may occur (Tzipori and Ward, 2002).

Cryptosporidiosis, as with many other infectious diseases, can be much more severe in immunocompromised patients such as those suffering from AIDS, undergoing chemotherapy or those that have genetic disorders compared with

immunocompetent patients. One of the earliest reports of cryptosporidiosis in humans was in an immunocompromised patient in 1976 (Meisel, 1976). Reports of cryptosporidiosis in people dramatically increased during the 1980s when the HIV AIDS epidemic began as the infected patients were unable to fight the infection. People suffering from HIV AIDS who have a $CD4^+$ T-cell count of less than 150/ml are very susceptible to severe persistent infection by *Cryptosporidium* with profuse and often life-threatening diarrhoea (Flanigan, 1994; Huang and Zhou, 2007). In immunocompromised patients infection is rarely confined to the small intestine and can spread to the heptobilliary tract, pancreatic duct and also the lungs (Denkinger et al., 2008; Lopez-Velez et al., 1995).

1.7.3 Immunity to disease

Despite the role of immune compromise in the development of disease in humans, the degree of adaptive immunity to infection and disease does not appear to be strong or of long duration (Okhuysen et al., 1998). Many people are infected whilst young and then again in adulthood (Current and Bick, 1989). A similar situation occurs for some primate species and broiler chickens, except that these hosts remain susceptible to infection but are protected against symptomatic disease (Current and Snyder, 1988; Miller et al., 1990). In farm livestock there is some evidence that animals will become less susceptible to disease the older they are (Harp et al., 1990; Kvac et al., 2014; Ortega-Mora and Wright, 1994) though one experimental study in calves demonstrated that it does not matter what age the calf is when it is first challenged with *C. parvum* the animal will shed oocysts and may have diarrhoea (Harp et al., 1990). However, if a calf has previous exposure to the parasite and is subsequently challenged then the animal will not shed oocysts (Harp et al., 1990). Similar studies in mice show that mice do not need to be exposed to the parasite to develop resistance at an older age (Harp et al., 1992; Harp et al., 1988), it has also been shown that the age at which the mouse becomes resistant to infection is also the age at which the intestinal flora is fully mature (Harp et al., 1992). It is not known if it is possible to develop immunity which is specific to a species or subtype of *Cryptosporidium* but which will not provide protection to infection with a different species or subtype. Most experimental trials have only tested the

development of immunity to a homologous challenge (Harp et al., 1990; Miller et al., 1990; Ortega-Mora and Wright, 1994).

1.7.3.1 Host Immune Response

Immunity to *Cryptosporidium* involves both the innate and adaptive immune response. Studies on this subject are few and the nature of the immune response is poorly understood.

The innate immune response will provide an immediate defence against the parasite invading the epithelial cells by preventing attachment. It has been shown that mucin interferes with the sporozoite's ability to attach to the cell wall (Barnes et al., 1998; Cevallos et al., 2000) and other immune cells such as natural killer (NK) cells will secrete cytokines which may have a direct effect on the parasite or signal a further immune response.

Following invasion of the host epithelial cells cytokines are produced which signal NK cells into action. When stimulated by TNFα and IL-12, NK cells produce IFN-γ (and other cytokines but mainly IFN-γ), NK cells also produce TNFß, which can directly damage target cells and cause cell lysis. TNFα activates APC (antigen presenting cells) such as dendritic cells and macrophages which are found in the villi of the intestinal mucosa. Activation of these leads to the secretion of further cytokines and interaction with $CD4^+$ T cells, these cells differentiate into either Th1 or Th2 cells. Th1 cells produce IL-12, IFN-γ and TNFß which enhance the cytotoxic response, Th2 cells produce other interleukins which stimulate the differentiation of B-cells into antibody producing cells (Figure 1-6).

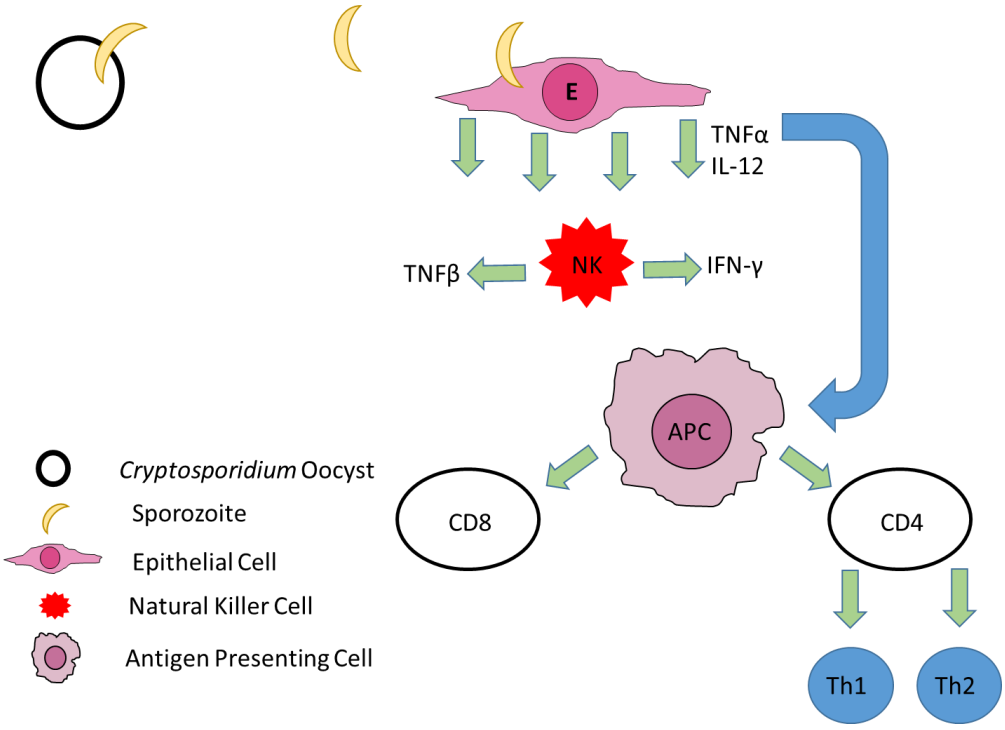


Figure 1-6: Simplified diagram showing the most important steps and interactions in the acquired immune response against *Cryptosporidium* infection.
Adapted from *Cryptosporidium* from molecules to disease.(Thompson et al., 2003)

Much of the knowledge of immune responses to *Cryptosporidium* has been gained through infection trials in murine models. There are some studies investigating the immune responses to *Cryptosporidium* in humans but very few in livestock hosts.

Experimental infections in mice have shown that Th1 cytokines, $CD4^+$ T cells and IFN-γ are essential for elimination of *Cryptosporidium* infection (McDonald and Bancroft, 1994; McDonald et al., 1996). Mice lacking $CD4^+$ T cells become seriously ill if infected compared with 'normal' mice, the same is assumed for humans as it is known that AIDS patients with low $CD4^+$ T cell counts suffer from more severe disease which can be fatal (Hoepelman, 1996; Lopez-Velez et al., 1995; Tali et al., 2011).

Cytokines that play a key role in both innate and adaptive immunity include IFN-γ. In mice it has been established that IFN-γ is a major player in controlling *Cryptosporidium* infection, not only in cell-mediated immunity but in early innate immune responses as well (Riggs, 2002; Theodos, 1998). Mice deficient in IFN-γ and other cytokines (IL-12 and IL-18) have increased susceptibility to

infection (Mead and You, 1998; Tessema et al., 2009). Similar results have been reported for HIV-negative patients with IFN-γ deficiency (Gomez Morales et al., 1999) and *in vitro* studies have demonstrated that in human cells IFN-γ directly prevents the parasite from invading host cells (Pollok et al., 2001).

1.7.4 Control of cryptosporidiosis

Cryptosporidiosis is a difficult disease to control, as the oocysts are very environmentally stable and can survive for long periods of time in cool, moist conditions, infected animals shed huge numbers of infective oocysts (Fayer et al., 2010a) and infection can pass through a group of susceptible hosts very quickly. The oocysts are also resistant to many disinfectants (Chalmers and Giles, 2010), there are no vaccines available to prevent the disease, and treatment options are limited and often rely on rehydration therapy.

1.7.5 Therapeutics

At present there are very few products licensed in the UK for the treatment or prevention of cryptosporidiosis in livestock or humans, the few products which are available are not very effective and in most cases will only reduce the duration of shedding and have little or no effect on immunocompromised patients.

1.7.5.1 Livestock

The only licensed treatment for cryptosporidiosis in calves is halofuginone lactate which affects invading parasite stages of *Cryptosporidium* (Jarvie et al., 2005). This drug is approved for use in both prevention and treatment of cryptosporidiosis in calves at a dose of 0.10 mg/kg of body weight per day for seven consecutive days. As a preventative measure the drug should be given within 48 h of birth and as a therapeutic, within 24 h of the onset of symptoms. Treatment with halofuginone lactate does not completely prevent or cure disease but it does reduce oocyst shedding and duration of diarrhoea (Jarvie et al., 2005; Lefay et al., 2001; Trotz-Williams et al., 2011). There is no licensed treatment for cryptosporidiosis in sheep, goats or pigs.

Several other chemotherapeutic agents have been tested for the treatment of cryptosporidiosis in livestock but none have resulted in a significant reduction in oocyst shedding; some antibiotics have shown efficacy against *Cryptosporidium* oocyst shedding, clinical disease and mortality in calves, lambs and goat kids but these are not registered for use in calves (Fayer and Ellis, 1993; Viu et al., 2000). A few coccidiostats, such as decoquinate have been tested against *Cryptosporidium* with limited or no reduction in oocyst shedding (Moore et al., 2003).

1.7.5.2 Humans

For human cases of cryptosporidiosis the only treatment available is nitazoxanide, which interferes with anaerobic metabolism of the parasite. Like halofuginone lactate in the treatment of cryptosporidiosis of livestock this drug cannot prevent or cure the disease but does reduce shedding of oocysts and severity of disease in immunocompetent patients (Rossignol et al., 2001). It has little effect on the severity of disease in immunocompromised patients (Amadi et al., 2002).

1.7.6 Vaccines

At present there are no vaccines available to prevent cryptosporidiosis in either farm livestock or humans. However, several attempts to develop such a vaccine have been made, some of which were partially successful under experimental conditions. Calves that were immunised with killed (gamma-irradiated or lyophilised) *C. parvum* oocysts showed a reduction in oocyst shedding and levels of diarrhoea compared to non-immunised calves (Harp and Goff, 1995; Jenkins et al., 2004). However, this vaccine was not effective when tested under field conditions (Harp and Goff, 1998).

Infection with *Cryptosporidium* often occurs within the first week of life so attempting to immunise the calves themselves is probably ineffective as this will not mediate a significant immune response prior to infection (de Graaf et al., 1999; Innes et al., 2011). Some attempts to immunise cows so that they produce antibodies against *Cryptosporidium* which can be passed to their calves in the colostrum have been made. Calves receiving colostrum from cows vaccinated

with a recombinant *C. parvum* were protected against diarrhoea and also a reduction in oocyst shedding compared to the calves which did not receive colostrum from vaccinated cows (Perryman et al., 1999).

1.7.7 Disinfectants

The oocysts are highly resistant to commonly used disinfectants (Virkon® S (peroxygen compounds (DuPont) (Ares-Mazas et al., 1997), FAM® 30 (Iodophors (Evans Vanodine)), Sorgene® 5 (hydrogen peroxide (BASF)) at their recommended working concentrations, (reviewed by Casemore & Watkins (, 1998)), making them difficult to eliminate in cattle areas on farms.Some less common or harder to obtain disinfectants (Keno^{TM}Cox (amine based (CID Lines)), (Ox-Virin & Ox-Agua (peroxygen compounds (Edificio OX)) have been shown to be effective against *Cryptosporidium* oocysts, at least *in vitro* (Castro-Hermida et al., 2006b; Naciri et al., 2011; Quilez et al., 2005). There are very few field trials testing these disinfectants but one such study demonstrated that even though these may be effective *in vitro* they are not effective enough to rely on for the effective elimination of *Cryptosporidium* oocysts on farms (Keidel and Daugschies, 2013).

1.7.8 Farm management

Most recommendations for controlling cryptosporidiosis rely on good management and hygiene practices as the oocysts are so difficult to eliminate it is better to try and reduce the environmental contamination in the first instance. Frequent mucking out of calving areas and calf houses combined with steam-cleaning and disinfection can help to reduce environmental build up (Castro-Hermida et al., 2002) as can thorough cleaning with hot water followed by drying (Harp and Goff, 1998) as the oocysts are susceptible to extremes of temperature and desiccation (Robertson et al., 1992).

Other calf management practices such as housing can have an effect on infection rates but some of the results are conflicting. In one study it was shown that housing calves with their dams decreased the risk of *C. parvum* infection while calves removed from their dam and kept in individual pens were at higher risk of infection (Duranti et al., 2009). In contrast, other studies have shown that the

opposite is true (Quigley, III et al., 1994). It is also possible for infection to be passed between subsequent calves in a particular pen (O'Handley et al., 1999) if the pen is not thoroughly cleaned between calves. Calves housed outdoors are at less risk than those housed indoors regardless of the housing arrangements (Quigley, III et al., 1994).

1.8 Summary

In summary, cryptosporidiosis is a very serious cause of enteritis and disease in many vertebrate and invertebrate species. Detailed, longitudinal studies in farm livestock are few and since the development of new molecular tools the dynamics of parasite infection should be further investigated. There is a lack of diagnostic tools to enable the quick speciation of *Cryptosporidium* present in calf samples; it is also difficult to detect mixed infections meaning that zoonotic infections can be missed where the predominant species is a less-pathogenic one. In particular, the transmission of *C. parvum* from adult cattle to neonatal calves is much debated and the ability of animals to develop immunity to disease is not well understood. This PhD work hopes to answer, in part, some of these important questions.

1.9 Aims

The main aims of this PhD are:

1. To develop new typing tools (multiplex PCR) to distinguish *Cryptosporidium* species commonly found in cattle samples

2. To assess the shedding profile of *Cryptosporidium* species and genotypes found in calves on a study farm

3. To assess the role of adult cattle in the transmission and persistence of *Cryptosporidium parvum* on a study farm

4. To assess the host-pathogen interactions involved in animals given serial experimental challenges with distinct *Cryptosporidium parvum* isolates

Chapter 2: Materials and Methods

The methods described here are general protocols used throughout this PhD in various studies. Animal and sample numbers are described in more detail in the relevant chapter as well as any adaptations or changes to the general protocol.

2.1 Cattle Sample Collection

2.1.1 Dairy Calves

Faecal samples from dairy calves were collected directly from the rectum using a gloved finger and bag for collection; where possible the entire motion was collected. It was not always possible to collect a sample from each calf on each sampling day; therefore the number of samples collected per calf did vary. After collection the samples were transferred to the laboratory where a sub-sample was aliquoted into a bijoux tube and stored at -20°C.

2.1.2 Adult Dairy Cattle

Collection of faeces from adult dairy cattle was carried out by observing the cow until she defecated and picking the sample from the floor; the whole pat was collected where possible. The cows were housed in cubicle sheds before calving, in straw courts at calving and in slatted cubicle sheds after calving when they returned to the milking herd. After collection the samples were transferred to the laboratory where the entire motion was mixed and a sub-sample aliquoted into a 125 ml tub and stored at 4°C.

2.2 Experimental Animals

All experimental animals were used in accordance with Home Office regulations, with approval of the experimental design sought before the study commenced from the Moredun Research Institute's ethics committee.

2.2.1 Lambs

Forty newborn (<24 h old) Texel and Texel cross male lambs were obtained from Firth Mains farm, Roslin.

2.2.1.1 Pre-amplification of *Cryptosporidium* isolates

Four of the 40 lambs were obtained one month prior to the study start date. These lambs were separated into two groups of two and each group was inoculated with one of two different *C. parvum* isolates (see section 3.3.1).

2.2.1.2 Study animals

Animals used in the study were separated into six groups of six depending on experimental challenge (see section 3.3.2).

2.2.2 Clinical Scoring

Lambs were monitored a minimum of three times per day for the first six weeks and then a minimum of twice daily for the remainder of the study. Daily monitoring involved the recording of the volume of milk each lamb drank along with how well the animal drank, the demeanour of the animal and the appearance of the faeces produced (Table 2-1).

Table 2-1: Scoring system used to score lambs daily

	Demeanour Rising	Demeanour Standing	Feeding	Faecal Consistency
Score	**Description**	**Description**	**Description**	**Description**
1	Already on feet Happy to rise	Fit and well	Feeding vigorously Complete feed with interruptions (due to excitement) Complete feed with assistance (due to age)	Solid Pellet (s)
2	Reluctant to rise	Listless	Partial feed with assistance (due to age)	Soft Pellet (s)
3	Needs assistance to rise	Unsteady on feet	Complete feed with interruptions (due to illness)	Soft Mass
4	N/A	Cannot remain standing	Complete feed with assistance (due to illness)	Scour
5	N/A	N/A	Partial feed – no assistance	Liquid
6	N/A	N/A	Partial feed with assistance (due to illness)	N/A
7	N/A	N/A	Animal refuses to feed	N/A

2.2.3 Blood Sampling

Blood samples were taken weekly from each lamb by jugular vein puncture using a vacutainer needle (BD Vacutainer, USA). Blood samples were left overnight at 4°C to clot then centrifuged at 2000 × *g* for 10 mins and the serum transferred to a 1.5 ml tube. Serum samples were stored at -20°C.

2.2.4 Faecal Collection

Total faecal output was collected from each lamb using a specially designed harness and bag system (see section 2.2.4.1) which the lamb wore continuously. This enabled the lambs to be penned in groups rather than separately but still allowed collection of samples from each lamb individually. This system also ensured that after inoculation the lambs were not exposed to any faecal matter and were not re-infected by oocysts shed in the faeces. Bags were taken off and replaced with a new one clearly marked with group/animal/sample when required. Samples were stored in the bags at 4°C until they were processed (see section 2.5.2).

2.2.4.1 Harness and bag design

An adjustable body harness with attachment points for the collection bag was designed and made using 20 mm webbing and plastic buckles (Figure 2-1). Collection bags were made using resealable polythene bags with a pipe cleaner to keep the bag open, a hole for the tail and a length of India cotton tape to attach the bag to the harness.

Figure 2-1: Lambs wearing harness and bags for collection of faeces.

2.3 *Cryptosporidium* oocysts

In the experimental study (Chapter 3) three different *C. parvum* isolates were used, these are described in more detail in section 3.3.3.

2.3.1 Collection of faeces for oocyst purification

Faecal samples were collected from calves on three different farms known to have a problem with *Cryptosporidium* (history of cryptosporidiosis diagnosed by a veterinarian and regular occurrence of scour in neonatal calves). Samples were collected using the method described in section 2.1.1 from 2-6 scouring calves on each farm.

2.3.2 Identification, quantification, extraction and purification of positive samples

2.3.2.1 Identification and quantification

Each faecal sample was weighed and the starting weight recorded. If the sample was not sufficiently liquid (easy to pipette through a 1000 µl pipette tip) then water was added and the sample re-weighed. Approximately 1 ml of the faecal suspension was added to a bijoux, weighed, and diluted 1:5 with dH_2O. The sample was then vortexed vigorously and 100 µl added to 900 µl malachite green stain (0.16% malachite green, 1% SDS). Using a haemocytometer 10 µl of the stained faecal suspension was examined under the microscope for the presence of oocysts. The number of oocysts in each of the four, sixteen grid corner squares were counted (Figure 2-2) on both side of the haemocytometer and the average number of oocysts calculated. The detection limit of this counting method is 62500 oocysts per gram of faeces.

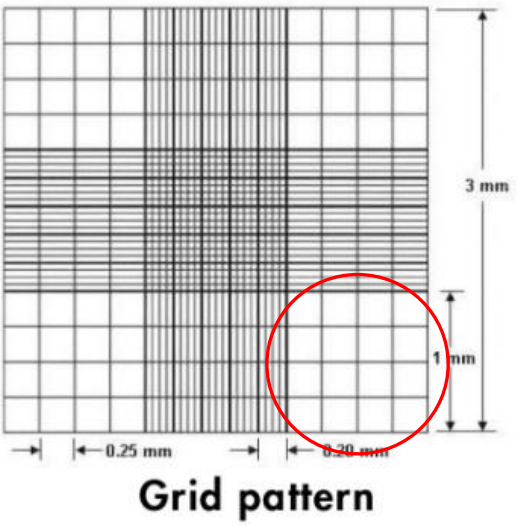


Figure 2-2: Example of the grid pattern on a haemocytometer.
The number of oocysts in each of the four sixteen grid squares (circled)

Quantification of the oocysts in each sample was done as follows (mean) × (dilution factor) × (10^4) × (sample weight).

Oocysts were extracted from positive samples with high oocyst numbers using the method described in section 2.3.2.2.

2.3.2.2 Extraction and purification of oocysts from positive samples

Oocysts were extracted from faeces that were found to be positive for *Cryptosporidium* when tested as described in section 2.3.2.1 using a sucrose flotation method. Sixty millilitres of faecal suspension was added to a 250 ml centrifuge bottle with 90 ml 45% sucrose solution and mixed thoroughly before being centrifuged at 1000 × *g* for 20 mins. The meniscus was removed and diluted 1:5 with dH_2O and centrifuged at 1000 × *g* for 20 mins. The supernatant was discarded and the pellet resuspended in 20 ml dH_2O in a 30 ml universal tube, this was centrifuged for 5 mins at 500 × *g*, the supernatant discarded and the pellet resuspended in 20 ml dH_2O. This step was repeated until the supernatant was clear (four washes on average). After the final wash the pellet was resuspended in 10 ml 10% sodium dodecyl sulphate (SDS) and left to stand at room temperature for 1 h. After 1 h the samples were centrifuged at 500 × *g* for 5 mins, the supernatant discarded and the pellet resuspended in 10 ml dH_2O the centrifugation and resuspension step were repeated. The samples were stood for 10-15 mins to allow any debris to flocculate and the supernatant removed to a clean universal tube. If any debris still remained after this step 100 µl 2% sulphuric acid was added to aid flocculation.

Once oocysts were extracted from the faeces they were counted using the method described in section 2.3.2.1 except 10 µl oocyst suspension was added to 990 µl 0.16% malachite green, 10% SDS.

2.3.3 Viability of oocysts and inoculum preparation

In order to test if the oocysts extracted from the calf faeces were infective a excystation assay was performed. A certain percentage of oocysts must be viable for the challenge dose to be effective.

2.3.3.1 Excystation assay

Forty microlitres of oocyst suspension was transferred to a 0.5 ml mini microcentrifuge tube and centrifuged at 12,500 × *g* for 30 s to pellet the oocysts. The supernatant was discarded and 40 µl of 1x Hanks Buffered Saline Solution (HBSS) was added and the pellet resuspended, 50 µl 1x trypsin (prepared in HBSS at pH3) was added and the sample mixed and incubated at 37°C for 1 h.

Following incubation the sample was centrifuged at 500 × *g* for 30 s and the supernatant discarded, the pellet was resuspended in 90 µl 1 × HBSS and 10 µl 2.2% sodium bicarbonate and 10 µl 1% sodium deoxycholate (in HBSS) added. The sample was mixed and incubated at 37˚C for 40 mins. Following incubation the sample was centrifuged at 12,500 ×*g* for 30 s and the supernatant discarded. The pellet was resuspended in 50 µl 3% gluteraldehyde to fix the sample. To assess the viability of the oocysts 10 µl fixed sample was spotted onto a microscope slide a cover slip placed on top and examined under × 40 phase contrast magnification. The total numbers of oocysts, sporozoites and empty oocyst shells were counted until the combined total reached 250. The sporozoite per shell ratio was calculated as follows:

$$\frac{\text{Sporozoite Count}}{\text{Shell Count}} = \text{Sporozoites per Shell}$$

And the excystation percentage as follows:

$$\frac{\text{Shell Count}}{\text{Oocyst Count} + \text{Shell Count}} \times 100 = \text{Percentage Excystation}$$

Each oocyst can contain a maximum of 4 sporozoites. A sporozoite per shell ratio of >2 and an excystation percentage of >85% are considered good indicators of viability. Figure 2-3 shows excysted oocysts and sporozoites.

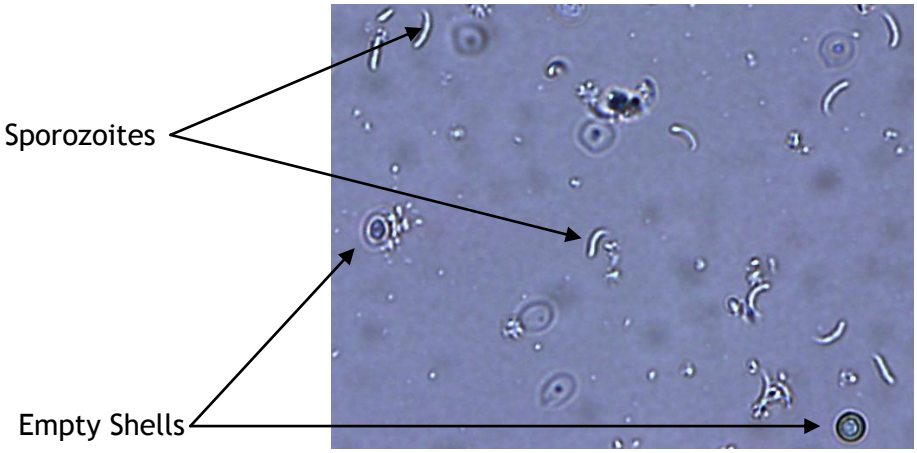


Figure 2-3: Excysted oocysts. Empty oocyst shells (dark circles) and sporozoites (bright "commas") can be seen.

2.3.3.2 Preparation of inoculum

Cryptosporidium oocysts were surface sterilised using 2 ml 70% EtOH (ethanol) for 30 mins before being resuspended in phosphate buffered saline (PBS). Oocysts were then counted and the oocyst suspension diluted with PBS to give the desired infective dose.

2.4 Sample Processing

2.4.1 Adult Dairy Cattle

A 50 g sub-sample of faeces was thoroughly mixed and added to a 1000 ml cylinder containing 600 ml water and 7 ml 2% sulphuric acid. The volume was then made up to 700 ml by the addition of water. The samples were mixed for 5 mins on a magnetic stirrer and then left to settle for 5 mins. The heavy, fibrous material sank to the bottom of the cylinder while the oocysts were suspended in the liquid fraction. Using a 25 ml pipette tip the supernatant was removed and added to two 250 ml centrifuge bottles, the bottles were then centrifuged at 1000 × *g* for 20 mins and the supernatant poured off. The thin layer of sediment at the bottom of the centrifuge bottle was resuspended in 7 ml dH_2O and poured into a 15 ml tube; two 3.5ml washes were used to remove the whole layer. The 15 ml tubes were centrifuged at 5000 ×*g* for 5 mins and the supernatant poured off. The pellet was then subjected to salt flotation to purify the oocysts further. Three grams of the pellet was added to 8 ml saturated salt solution and

thoroughly mixed by vortexing then 2 ml dH_2O trickled on top of the salt solution before centrifugation at 1000 × g for 8 mins. Following centrifugation the water layer was gently swirled using a Pasteur pipette to create a vortex which draws the oocysts from the layer between the dH_2O and salt into the dH_2O, the layer was removed and added to 6 ml dH_2O, the total volume was made up to 10 ml, mixed by inverting and centrifuged at 5000 × g for 5 mins. The supernatant was poured off and the pellet resuspended in TE buffer (10 mM Tris-HCl, 0.5 mM EDTA) for DNA extraction.

2.4.2 Experimental Lambs

Samples collected from the lambs used in the experimental challenge (Chapter 3) were processed individually. The sample was weighed and the weight recorded, water was then added to the sample to make the faeces liquid enough to pipette. If the sample was already liquid then no additional water was added. The sample was then re-weighed and the new weight recorded. Using a food processor the sample was homogenised completely and a 2 ml sample aliquoted into a tube for storage for subsequent use. These aliquots were stored at -20°C. A small subsample ~850 µl was taken and used to quantify the oocysts present in the sample (see section 2.3.2.1). The remainder of the faecal material was discarded.

2.5 DNA Extraction

2.5.1 Adult Cattle Faecal Samples

DNA was extracted using a Macherey-Nagel Tissue Kit (Macherey-Nagel, Germany) following the manufacturer's instructions except 10 × freeze-thaw cycles in liquid nitrogen were added. Briefly, the resuspended pellet (see section 2.4.1) was mixed vigorously using a vortex then centrifuged at 4,000 × *g* for 15 mins. The supernatant was poured off and the pellet resuspended in 200 µl buffer T1, 10 × freeze-thaw cycles in liquid nitrogen were performed and 25 µl proteinase K (provided in the kit) added. The samples were then incubated at 56°C overnight. Following incubation the samples were briefly mixed using a vortex and then 200 µl lysis buffer B3 was added and incubated at 70°C for 10 mins, after incubation samples still containing insoluble particles were

centrifuged at 11,000 × *g* for 5 mins and the supernatant transferred to a new 1.5 ml tube. The samples were mixed by vortexing and the binding conditions adjusted by adding 210 µl EtOH (100%). The whole sample was loaded into a spin column placed in a 2 ml collection tube, the samples were centrifuged at 11,000 × *g* for 1 min, the flow through discarded and the spin column placed into a new 2 ml collection tube. The silica membrane was washed twice, first using 500 µl wash buffer BW and centrifugation for 1 min at 11,000 × *g* and secondly using 600 µl wash buffer B5 followed by centrifugation for 1 min at 11,000 × *g*. After the initial wash the spin column was placed in a new 2 ml collection tube and after the second wash the flow through was discarded and the samples centrifuged at 11,000 × *g* for 1 min to dry the silica membrane. The DNA was eluted in 100 µl dH_2O and stored at -20°C.

2.5.2 Calf and Lamb Faecal Samples

DNA was extracted from calf and lamb faecal samples in much the same way as described for adult samples (section 2.5.1) except 200 µg (or 200 µl if liquid) of faecal material was added to TE buffer and no freeze-thaw cycles were performed as previous work in the laboratory had shown that free-thawing of faeces from calves or lambs did not improve the detection of *Cryptosporidium*.

2.6 Polymerase Chain Reaction (PCR)

2.6.1 *Cryptosporidium* Specific 18S rRNA PCR

A previously described nested PCR protocol (Xiao et al., 1999) was used to amplify a ~840 bp fragment of the 18S rRNA gene. Each 25 µl reaction contained 10 × PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 4.4 µM EDTA, 113 µg ml-1 BSA, 1 mM each of the four deoxyribonucleotide triphosphates) (MacLeod et al., 1999), 0.5 units BioTaq (Bioline, UK), 10 µM of forward and reverse primers (Table 2-2) and 3 µl DNA in the primary round and 1 µl primary PCR product in the secondary round. The total volume was made up to 25 µl with dH_2O. In each PCR run one set of positive control DNA and negative controls consisting of dH_2O were included. All PCR reactions were carried out in triplicate.

Table 2-2: Primer sequences and size of amplicon when used with primer pair for the 18S rRNA gene.

Primer	Sequence 5'-3'	Fragment Size (bp)
AL1687(EF)	TTCTAGAGCTAATACATGCG	1370
AL1691(ER)	CCCATTTCTTCGAAACAGGA	
AL1598 (IF)	GGAAGGGTTGTATTTATTAGATAAAG	840
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA	

Cycling conditions were 3 mins at 94°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C and 1 min at 72°C. The final extension was 7 mins at 72°C. Secondary amplification products (~3 µl) were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000.

2.6.2 Nested Species Specific Multiplex PCR (nssm-PCR)

For the differentiation of *Cryptosporidium* species, the primary PCR products from the 18S rRNA nested PCR were amplified using a nested species specific multiplex PCR (nssm-PCR) (Chapter 4) which can distinguish the four most commonly detected bovine species of *Cryptosporidium*. The primary PCR products were diluted with 50 µl dH_2O and 1 µl of the dilution used as template in the secondary round. Secondary PCR reactions contained 2.5 µl 10 × PCR buffer (section 2.6.1), 0.5 units BioTaq (Bioline, UK), 10 µM of each primer (

Table 2-3) and 1 µl template. Cycling conditions were 3 mins at 94°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 56°C and 1 min at 72°C. The final extension was 7 mins at 72°C. Secondary PCR products (~2 µl) were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000.

Table 2-3: Primer sequences and size of amplicon for nssm-PCR.
The *C. ryanae* specific primer contains a degenerate base which is shown in bold and underlined. (CaF: *C. andersoni* specific forward primer, C.rF: *C. ryanae* specific forward primer, C.phF: *C. parvum* specific forward primer, CbF: *C. bovis* specific forward primer.

Primer	Sequence 5'-3'	Fragment Size (bp)
AL1598 (IF)	GGAAGGGTTGTATTTATTAGATAAAG	840
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA	
CaF	GCAAATTACCCAATCCTGAC	625
CrF	TGTTAATTTTTATATACAAT**R**CTACGG	415
CphF	AGAGTGCTTAAAGCAGGCATA	241
CbF	CTTCTTATTCCTTCTAGAATAAAAATG	305

2.6.3 *gp60* Subtyping

A nested PCR designed to amplify a 450 bp fragment of the gp*60* gene (Brook et al., 2009) was used on a selection of *C. parvum* positive samples to identify the subtype. Each 25 µl reaction contained 10 × PCR buffer (section 2.6.1), 0.5 units BioTaq (Bioline, UK), 10 µM of forward and reverse primers (Table 2-4) and 3 µl DNA in the primary round and 1 µl primary PCR product in the secondary round. Cycling conditions were 3 mins at 94°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C and 1 min at 72°C. The final extension was 7 mins at 72°C. Secondary PCR products (~2 µl) were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000.

Table 2-4: Primer sequences and amplicon sizes for *gp60* PCR

Primer	Sequence 5'-3'	Fragment Size (bp)
GP60 1F (EF)	ATAGTCTCCGCTGTATC	480
GP60 1R (ER)	GAGATATATCTTGGTGCG	
GP60 2F (IF)	TCCGCTGTATTCTCAGCC	~375
GP60 2R (IR)	CGAACCACATTACAAATGAAG	

2.7 Sequencing

2.7.1 Purification of PCR products

PCR products which were sent for sequencing were purified using the Promega Wizard SV gel and PCR Clean-Up System (Promega, UK) following the manufacturer's instructions. Briefly, an equal volume of membrane-binding solution was added to the PCR in a 1.5 ml tube before being added to the minicolumn assembly. The solution was incubated at room temperature for 1 min then centrifuged at 16,000 × *g* for 1 min. The supernatant was discarded and the minicolumn reinserted into the collection tube. Seven hundred microlitres of membrane wash solution was added and the assembly centrifuged at 16,000 × *g* for 1 min. The supernatant was discarded and the wash step repeated with 500 µl membrane wash solution and centrifugation at 16,000 × *g* for 5 mins. The supernatant was discarded and the minicolumn centrifuged for a further min to remove all residual ethanol. The minicolumn was inserted into a clean 1.5 ml tube and the DNA eluted in 50 µl dH_2O.

2.7.2 DNA quantification

DNA concentration of purified PCR products was quantified using a NanoDrop ND1000 spectrophotometer (Labtech International Ltd, UK).

2.7.3 Sequencing

Sequencing of PCR products was carried out by GATC (GATC, Germany) using their "Light-run" service. Five microlitres of purified PCR product was sent at a concentration of between 20-80 ng/µl with 5 µl of the appropriate primer at a concentration of 5 pmol/µl. In some circumstances, instead of the light-run service, unpurified PCR products were sent to GATC for sequencing using their "Supremerun96" sequencing option which includes PCR purification. With this service 20 µl PCR product was sent with a separate 1.5 ml tube containing 50 µl of the appropriate primer at a concentration of 10 pmol/µl.

2.7.4 Sequence analysis

Sequences were analysed using BioEdit Sequence Alignment Software (www.mbio.ncsu.edu/bioedit/bioedit.html) and Chromas Lite (www.technelysium.com).

2.8 Statistical Analysis

Statistical analyses were performed using Minitab17 (Version 17.1.0.0, Minitab Inc.) $P \leq 0.05$ was considered significant. Survival curves were produced using R statistical package.

For association between binary outcome variables (Chapter 3) , such as the presence/absence of diarrhoea, chi squared tests were used for univariate analysis and logistic regression for multivariate analysis. Continuous outcomes, such as number of days on which diarrhoea occurred, were tested for association with the explanatory variables using ANOVA. For the outcome variable of first day of diarrhoea, data was right censored (i.e. no values were recorded if the lamb did not develop diarrhoea) and Kaplan–Meier survival curves were analysed.

Chapter 3: Can infection with one isolate of *Cryptosporidium parvum* protect against infection with another?

3.1 Introduction

An experimental challenge in neonatal lambs was carried out to determine if lambs are able to develop immunity to infection with *C. parvum* following a primary infection. We also investigated the effect of an increase in age on susceptibility to disease when older naïve lambs were challenged with *C. parvum*.

There are very few studies which have investigated the effect of sequential infection with distinct strains of *C. parvum* on neonatal livestock. Two studies, one in sheep (Ortega-Mora and Wright, 1994) and one in cattle (Harp et al., 1990) have studied the effect of age on infection with *Cryptosporidium* species (believed at the time to be *C. parvum*). In the study in sheep, they noted an age-related susceptibility to infection with younger animals (<4 weeks of age) shedding larger numbers of oocysts for a more prolonged period compared with those infected a few weeks later. Both of these studies showed that animals exposed to the parasite at a younger age do not have such severe disease when exposed a second time showing that the animals were able to develop immunity to a homologous challenge. Most of the studies done in animals investigating immune responses to infection were carried out over 10 years ago before the discovery of some of the different species of *Cryptosporidium* which are now known to infect farm livestock. At this time there was not the knowledge of different isolates of *C. parvum* that we have now. It is not known if infection with one isolate of *C. parvum* will provide protection against infection with another isolate. It is possible that if one isolate does provide protection against another that there is potential for development of control measures using a less virulent (if one is identified) isolate to protect against infection from severe disease caused by a more virulent subtype. This strategy has been used previously to protect against other protozoan parasites, for example, the vaccine Toxovax® (MSD, Animal Health) uses a less virulent strain (S48) of the *Toxoplasma gondii* parasite to provide protection to pregnant ewes from more virulent strains of *T. gondii*.

Similar studies have been carried out in other host species with different *Cryptosporidium* species. Kvac et al (2014) investigated the age-related susceptibility of pigs to *C. scrofarum*; previous work had indicated that while *C. suis* (the other pig adapted *Cryptosporidium* species) infected all age groups of pigs it appeared that *C. scrofarum* only infected older animals. In their study the authors infected groups of pigs ranging from 4 – 8 weeks of age with *C. scrofarum* oocysts and tested all faecal samples produced from each animal. The presence of *Cryptosporidium* oocysts was confirmed using aniline-carbol-methyl-violet (ACMV) staining and 18S nested PCR.

The results of the study showed that *C. scrofarum* was not detected in the faeces of 4 week old pigs while in pigs aged 5-8 weeks, oocyst shedding was detectable by both microscopy and PCR from 6-8 DPI. The authors suggested that the animals became more susceptible to invading sporozoites at weaning when the gut was adapting to different food.

Another study investigating *Cryptosporidium* infection in chickens demonstrated an age-related susceptibility to infection (Hatkin et al., 1993). Chicks infected with *C. baileyi* at 1 day of age showed severe clinical disease (diarrhoea, respiratory distress and lesions detected by necropsy) and 28.7% (n=33) of the infected birds died. Naïve birds infected at 7 days of age had less severe clinical disease with a longer prepatent period and naïve birds infected at 14 days of age showed only asymptomatic oocyst shedding.

Some studies investigating other protozoan parasites have carried out similar trials and one such trial showed that, similarly to the age-related susceptibility of pigs to *C. scrofarum,* rabbits older than 19 days were susceptible to *Eimeria intestinalis* whereas younger rabbits (up to 16 days of age) were resistant to infection (Pakandl and Hlaskova, 2007).

In piglets, coccidiosis caused by *Isospora suis* is responsible for up to 20% of cases of piglet diarrhoea (Lindsay et al., 1985). One study carried out by Koudela (1999) examined the effects of age and sequential infection with *I. suis* in piglets.

Naïve piglets infected at 3 days of age showed clinical signs of disease (diarrhoea) at 2-4 DPI which lasted until 11 DPI following the primary challenge. Piglets which were re-challenged showed less severe clinical signs (less diarrhoea) at 5 d post-secondary challenge, and the piglets which were infected for the first time at 19 days of age showed similar clinical signs to piglets which were re-challenged. The authors suggested that this demonstrated that age has more of an effect on development of resistance to *I. suis* than acquired immunity by previous exposure.

The trial described in this chapter aims to answer the question of whether lambs are able to develop immunity following infection with *Cryptosporidium parvum* and if this will protect them against a homologous or heterologous infection with *C. parvum*. The effects of increasing age on development of resistance to disease will also be explored. The possibility of differences in isolate pathogenicity/virulence will also be examined.

3.2 Aims

1) Determine whether lambs develop immunity following a primary infection with *C. parvum* to a homologous or heterologous challenge

2) Determine the effects of an increase in age on the resistance or susceptibility to infection by *C. parvum*

3) Determine if there are differences in the severity of disease caused by distinct *C. parvum* isolates

3.3 Materials and Methods

3.3.1 Animals

Forty Texel and Texel cross male lambs were obtained from Firth Mains farm, Roslin at <24 h of age and were housed in groups and kept *Cryptosporidium*-free until challenge.

3.3.2 Experimental Design

A total of 40 lambs were used in the experiment and were separated into eight groups. Four lambs were used for pre-amplification of two different *C. parvum* isolates to produce oocysts for inoculation, these lambs were separated into two groups each containing two lambs (Table 3-1).

Table 3-1: Pre-amplification of *C. parvum* isolates.

Group	*C. parvum* Isolate	Challenge	Number of Animals
Group A	BB	1×10^5	n=2
Group B	LH	1×10^5	n=2

The remaining 36 lambs were used for the experimental trial and were separated into six groups each containing six lambs. Group I and II were age-related susceptibility challenge groups (not challenged until week 6), Group III and VI were homologous challenge groups and Group IV and V were heterologous challenge groups (Figure 3-1).

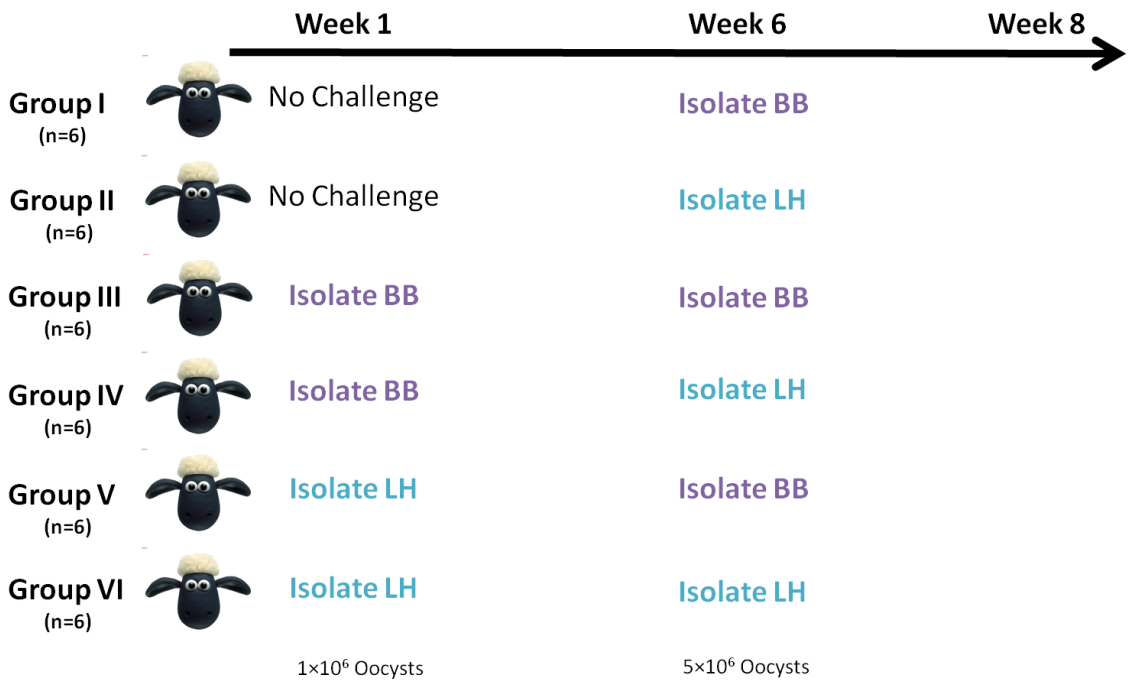


Figure 3-1: Experimental design.
Lambs were infected with two different *C. parvum* isolates at either 6 weeks of age only or at <1 week of age and re-infected with a homologous or heterologous challenge at 6 weeks of age.

3.3.3 *Cryptosporidium parvum* isolates

C. parvum isolates were obtained from calves from three dairy farms (Farm OV, Farm BB & Farm LH) in Scotland with known histories of cryptosporidiosis and assessed for suitability for use in the trial. Isolates from two farms (Farm BB & Farm LH) were chosen for use in the experimental trial.

The isolates used in the experimental trial were obtained from naturally infected calves on two dairy farms; one in Midlothian (also the study farm used in the studies described in Chapter 5 & 6 (Farm LH), Scotland and the other in the Scottish Borders (Farm BB), Scotland. In general the farm management practices on both farms was very similar. Farm LH milked approximately 240 dairy cows (Holstein Friesian and Norwegian Red) and farm BB milked approximately 500 dairy cows (Holstein Friesian). Both used artificial insemination (AI) to impregnate their cows, any cows which did not hold to AI were serviced naturally by the stock bull, on farm LH only heifers not holding to AI were serviced naturally. On both farms the only animals bought in was a new stock bull every 3-4 years.

For prevention of enteritis, in-calf cows and heifers were vaccinated with Rotavec^{TM} Corona vaccine (MSD Animal Health, UK) in accordance with the manufacturers recommendations.

In-calf cows and heifers were removed from the main herd before calving and are housed in cubicle sheds with automatic scrapers. Approximately 1 week prior to calving the cows and heifers were moved to a straw bedding court, the pens were regularly bedded (~3 × per week) with fresh straw and are mucked out and steam cleaned annually. On farm LH once the cows calved the calf was kept with its dam for a minimum of 24 h and was assumed to have received adequate colostrum if the calf is seen to suckle. On farm BB the stockworker was alerted to all newborn calves within 1 h of birth and they ensured that each calf received an adequate volume of colostrum in the 2 h following birth. Farm BB had a frozen supply of colostrum with 2 litres ready at anytime to give to a calf if the dam did not have a sufficient supply or if the calf was born to a heifer. Only cows colostrum is used to feed newborn calves on farm BB as it is higher in antibodies (Morrill et al., 2012) than colostrum from heifers and the stockworker believes that this is beneficial to the calves.

Following birth, calves on farm LH were kept in small groups (3-5 calves) for 5 days and were bottle fed calf milk replacer before being moved to a bigger pen of ~20 calves. The calves were housed in a large straw bedded pen in a well ventilated barn and had free access to hay, water and pelleted calf feed. The calves were fed milk replacer through an automated system which dispenses a set volume of milk for each individual calf. The volume of milk is set by the stockworker. The calves were weaned at 3 months and were then moved to a larger indoor straw bedded pen of ~40 calves until they reach 6 months of age. At this point, depending on the time of year they were either turned out to pasture or moved to a cubicle shed until turn out. The straw courts were regularly bedded (~3 × per week) with fresh straw and were mucked out, steam cleaned and disinfected between calf batches. Each year when the calves were turned out the calf pens were thoroughly cleaned, disinfected and painted.

Calves on Farm BB suffering from mild or asymptomatic infection whilst still shedding large numbers of oocysts Calves on farm BB were kept in individual calf pens for 2 days following birth and, depending on the condition of the calf and

the weather conditions the calves may have had a calf jacket (Cosy Calf, UK) put on. The calf wore the jacket until the temperature was warm enough to remove the jacket. Whilst in individual pens the calves were bottle fed milk replacer. The individual pens were mucked out and disinfected between calves.

After 2 days the calves were removed from the individual pen and were housed in a straw bedded pen in a large well ventilated barn with approximately 15 other calves of a similar age group (<1 month). The calves were fed milk replacer through an automated system which dispenses a set volume of milk for each individual calf. The volume of milk was set by the stockworker. They also had free access to hay, water and pelleted calf feed. Once the calves were old enough they were moved into an adjacent pen with other older calves until weaning at 3 months. At weaning calves were either turned out or moved to a shed until turn out, depending on the time of year. The straw pens were regularly bedded (~3 × per week) with fresh straw and were mucked out, steam cleaned and disinfected between calf batches. Each year when the calves were turned out the calf pens were thoroughly cleaned and disinfected.

Faecal samples were collected directly from the rectum from calves (n=2-6) on each farm as described in section 2.1.1.

3.3.4 Analysis of Calf Faecal Samples

Following sample collection the oocysts present in each sample were counted as described in section 2.3.2.1 and DNA extracted from the faeces following the protocol in section 2.5.2.

3.3.4.1 Microsatellite PCR primers and conditions

A nested PCR protocol was used to amplify regions of five previously described microsatellite markers (Morrison et al., 2008). Each 20 µl reaction contained 10 × PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 4.4 µM EDTA, 113 µg ml-1 BSA, 1 mM each of the four deoxyribonucleotide triphosphates), 0.5 units BioTaq (Bioline, UK), 10 µM of forward and reverse primers (Table 3-2) and 1 µl DNA in the primary round and 1 µl primary PCR product diluted 1:100 in the secondary round. In the secondary round 10 µM primers labelled with FAM or HEX were used (Table 3-2). The total volume was

made up to 20 µl with dH_2O. In each PCR run one set of positive control DNA and negative controls consisting of dH_2O were included.

Table 3-2: Primers used for microsatellite analysis of *Cryptosporidium* isolates

Primer	Sequence 5'-3'
MM5 (EF)	TCACAAGTTACCCCTTCTGATGCTG
MM5 (ER)	TCCACCTCCGGATTGGTTGTG
MM5 (IF)	CCTGGACTTGGATTTGGACTTACACC
MM5 (IR)	GGAGAAGATAAGCTAGCCGAATCT
MM18 (EF)	GTTCAGCTGATACGGGTTTGCAACA
MM18 (ER)	CATCACCATCTCCTCCGCCAGA
MM18 (IF)	CTTTCTGGAGGGTTTGTTCCTCC
MM18 (IR)	CTTCCTGATGATCCAGGCCAAGC
MM19 (EF)	TGGTTTTAGCTAAGGAAGCGATAG
MM19 (ER)	CTGCTGCTGCTGTTGCTTTA
MM19 (IF)	GATTCTGTCAACTTTGAATTCAG
MM19 (IR)	CCAACCCCGAATTCATTTCCAAC
TP14 (EF)	GAGAAGGAGCAATGGGAGCA
TP14 (ER	TCCTCCTTTTTGCCCTTGAA
TP14 (IF)	CTAACGTTCACAGCCAACAGTACC
TP14 (IR)	CAATAAAGACCATTATTACCACC
MS1 (EF)	AAGGGTGAGGATGAGCAGAA
MS1 (ER)	TTCTTAACTTTCCATTTTGAGTGA
MS1 (IF)	TTAGTCGACCTCTTCAACAGTTGG
MS1 (IR)	GGAACACCATCCAAGAACCAAAGGT

Secondary amplification products were visualised following electrophoresis on a 2% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000).

3.3.4.2 Microsatellite Analysis and Allele Identification

Labelled PCR products were subjected to fragment analysis using capillary electrophoresis (CE) via ABI 3730 (Applied Biosystems; University of Dundee), using size standard Genescan ROX500 (Applied Biosystems). A subset of samples was also re-sized using ROX400HD (Applied Biosystems). Trace files were then analysed using both Peak Scanner (Applied Biosystems) and STRand (www.vgl.ucdavis.edu/informatics/strand).

3.3.4.3 *gp60* subtyping

Subtyping of *C. parvum* positive samples was carried out by amplification and sequencing of the gp*60* gene (see sections 2.6.3 and 2.7). The PCR products were visualised on a 1.5% agarose stained with GelRed (Biotium, UK). Reactions were originally carried out in duplicate and if amplification was not successful the reactions were repeated. A sample was considered positive if a band corresponding with ~375 bp was seen. Positive, negative and extraction controls were included for each set of reactions. A positive result was only accepted when all negative controls were negative and all positive controls were positive.

gp60 positive samples were sequenced in the forward and reverse orientation by GATC Biotech (GATC, Germany) as described for "supremerun96" sequencing in section 2.7.3. The sequences were analysed using Chromas Lite (version 2.01 Technelysium) and the subtypes named as according to (Sulaiman et al., 2005)

3.3.5 Purification from faeces and preparation of inoculum

Oocysts were purified from faecal samples from calves from two farms (Farm BB & Farm LH) by selecting the samples with the highest oocyst counts in the smallest volume of faeces as determined by malachite green staining and microscopy as described in section 2.3.2.1. Using sucrose flotation (section 2.3.2.2) oocysts were purified from the *Cryptosporidium* positive faeces, the viability of the oocysts was tested by carrying out an excystation assay (section 2.3.3.1) which mimics the gut conditions and stimulates the oocysts (if viable) to excyst.

To prepare the oocysts for inoculation they were surface sterilised using 2 ml 70% EtOH for 30 mins before being resuspended in PBS. Oocysts were then counted (section 2.3.2.1) and the oocyst suspension diluted with PBS to give the desired infective dose.

3.3.5.1 Pre-amplification in lambs

To produce sufficient oocysts to inoculate all lambs in the experimental trial two of the *C. parvum* isolates (BB & LH) were amplified by orally infecting four neonatal lambs (in two groups) with 1×10^5 *C. parvum* oocysts in 1 ml PBS. Each

group of lambs were housed in separate plastic-sided lamb crates within individual concrete stables for ~13 days and were fed and monitored three times per day. Clinical assessments were made at each feeding/monitoring point using the criteria described in section 2.2.2. Total faecal output was collected from each lamb as described in section 2.2.4 using a harness and bag system. Faecal samples containing sufficient numbers of oocysts were processed as described in section 2.3.2 and processed to produce inoculum for the experimental trial.

3.3.6 Experimental Animals

Lambs used in the experimental trial were assigned to one of six groups. Each group of lambs was housed in concrete sided isolation boxes bedded with straw, the lambs were fed on UHT-treated milk three times per day via bottle and teat in a specially designed lamb feeder (Figure 3-2) to ensure that each lamb could access only its own milk. This enabled the handler to accurately record the feed intake of each individual animal; it also made the task of feeding 36 lambs slightly easier.

Figure 3-2: Lambs in specially designed feeder.
Lambs were fed UHT-treated milk three times per day in a specially designed feeder which allowed each lamb to be fed individually.

Lambs were fed exclusively on UHT-treated milk for the first two weeks of life with *ad libitum* access to hay and water, after this they were fed UHT-treated milk three times per day with *ad libitum* access to hay, water and pelleted lamb creep feed. Once the lambs reached six weeks of age they were fed UHT-treated milk twice per day and had *ad libitum* access to hay, water and pelleted lamb creep feed.

3.3.7 Collection of samples

3.3.7.1 Faecal Collection

Total faecal output was collected from each lamb using a specially designed harness and bag system (see section 2.2.4.1) which the lamb wore continuously (Figure 3-3). This enabled the lambs to be penned in groups rather than separately but still allowed collection of faeces from each lamb individually. This system also ensured that after inoculation the lambs were not exposed to any faecal matter and were not re-infected by oocysts shed in the faeces. Bags were taken off and replaced with a new one clearly marked with group/animal/sample when required. Samples were stored in the bags at 4°C until they were processed (see section 2.4.2).

3.3.7.2 Blood Sampling

Blood samples were taken weekly from each lamb by jugular venipuncture using a vacutainer needle (BD Vacutainer, USA). Blood samples were left overnight at 4°C to clot then centrifuged at 2000 × *g* for 10 minutes and the serum transferred to a 1.5 ml tube. Serum samples were stored at -20°C. Serum samples will be used to monitor the level of specific *Cryptosporidium* antibodies in an ELISA. Due to time constraints it was not possible to develop an ELISA during the course of this PhD.

Figure 3-3: Lambs with the harness and bag system to collect faeces
Lambs wore a harness and bag at all times to ensure that total faecal output was collected and the animals were kept away from oocysts which were shed in the faeces

3.3.8 Clinical Assessment

Lambs were monitored a minimum of three times per day for the first six weeks and then a minimum of twice daily for the remainder of the study. Daily monitoring involved the recording of the volume of milk each lamb drank along with how well the animal drank, the demeanour of the animal and the appearance of the faeces produced, see section 2.2.2 for more details.

3.3.9 Sample Processing

Samples collected from the lambs were processed individually. The sample was weighed and the weight recorded, water was then added to the sample to make the faeces liquid enough to pipette. If the sample was already liquid then no additional water was added. The sample was then re-weighed and the new weight recorded. Using a food processor the sample was homogenised completely and a 2 ml sample aliquoted into a tube for storage for use in DNA extraction. These aliquots were stored at -20°C. A small subsample ~850 µl was

taken and used to quantify the oocysts present in the sample (see section 2.3.2.1). The remainder of the faecal material was discarded (Figure 3-4).

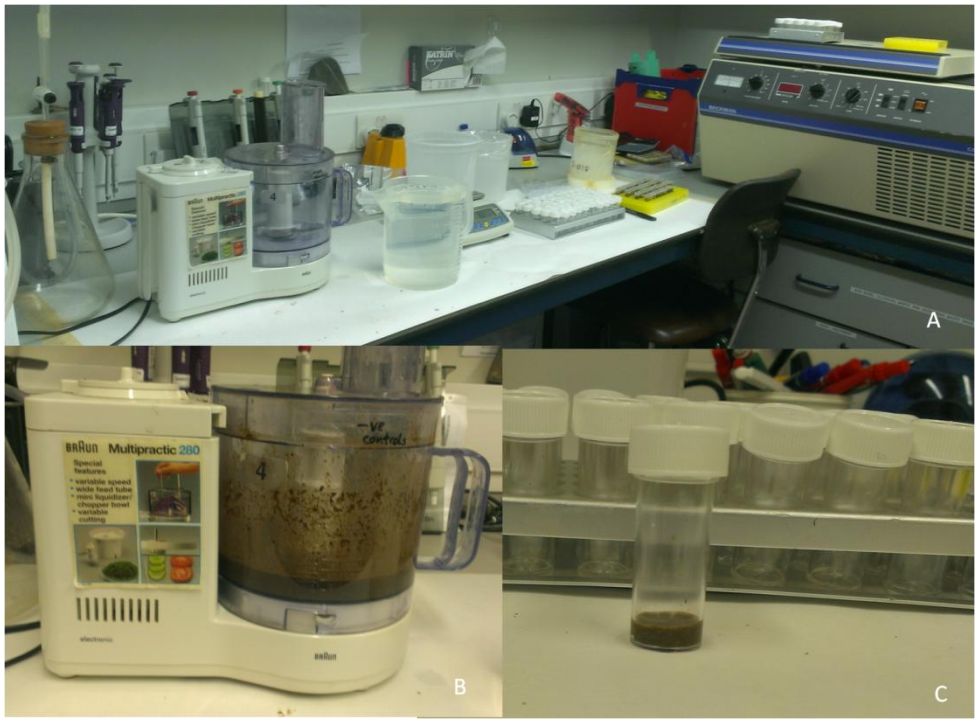


Figure 3-4: Sample processing
Entire faecal samples from each lamb were processed in the lab (A), the entire motion was homogenised in a food blender (B) and a sub-sample taken for counting of oocysts (C)

3.3.9.1 Oocysts counts

Oocysts present in the faeces were counted by staining the faecal suspension with 0.16% malachite green and examination under × 40 magnifications on a haemocytometer as described in section 2.3.2.1. A selection of samples from each lamb were counted; every sample produced by the lamb during the clinical stage of infection and 1-2 samples per week in the non-clinical stages were processed and counted.

3.3.10 Statistical Analysis

All statistical analyses were performed using Minitab17 (Version 17.1.0.0, Minitab Inc.). Outcome and explanatory variables are shown in (Table 3-3: Outcome and explanatory variables). Survival curves were produced using R statistical package.

Table 3-3: Outcome and explanatory variables
Codes used for definitions of value ranges and analytical approaches used for outcome and explanatory variables

	Code	Description	Value	Analysis
Outcome Variables	D1	Diarrhoea occurring during the time period wk1 to wk4	Yes/No	Chi-Squared test for univariate and logistic regression for multivariate
	D2	Diarrhoea occurring during the time period wk6 to wk8	Yes/No	Chi-Squared test for univariate and logistic regression for multivariate
	FD1	First day of diarrhoea during time period wk1 to wk4	1-28	Survival curve - Kaplan-Meier estimate
	FD2	First day of diarrhoea during time period wk6 to wk8	42-56	Survival curve - Kaplan-Meier estimate
	DD1	Number of days on which diarrhoea occurred in the time period wk1 to wk4	0-28	One-way ANOVA
	DD2	Number of days on which diarrhoea occurred in the time period wk6 to wk8	0-28	One-way ANOVA
	OC1	Total oocyst count for time period wk1 to wk4	0-1.8E^{9}	Logn transformed then One-way ANOVA
	OC2	Total oocyst count for time period wk6 to wk8	0-1.8E^{9}	Logn transformed then One-way ANOVA

	Code	Description	Value
Explanatory Variables	Age	Age of primary infection	D7, D42
	Isolate	Isolate used for challenge	BB, LH
	Dose	Challenge dose	Low, High
	Challenge	Previous exposure or not	Primary, Secondary
	Type	Secondary challenge with same isolate or not	Homologous, Heterologous
	Group	Group to which lamb was assigned	I - VI
	Animal	Unique ID within Group	A-F

3.3.10.1 Clinical Disease

The association between clinical disease (D1, D2, FD1, FD2, DD1 & DD2) for the dependant variables was analysed using chi-squared test, binary logistic regression and One-way ANOVA. The data for FD1 and FD2 was right-censored and survival curves were produced using R statistical package. The interval to morbidity (diarrhoea) was tested for each isolate using the "survdiff" function of R, using the G-rho family of Harrington and Fleming (1982). Weights on each morbidity are *S(t)^rho,* where *S* is the Kaplan-Meier estimate of survival, with rho = 0, this is the log-rank or Mantel-Haenszel test, and with rho = 1, it is equivalent to the Peto & Peto modification of the Gehan-Wilcoxon test.

3.3.10.2 Oocyst Shedding

Logn transformation was applied to all oocyst count data which resulted in approximately normal distributions, according to Anderson-Darling tests for normality, taken together with visual inspection of histograms of transformed data. One-way ANOVA with Tukey's post-hoc test was used to explored the association with explanatory variables.

3.4 Results

3.4.1 *Cryptosporidium parvum* isolates

3.4.1.1 Microsatellite analysis to identify suitable isolates

Microsatellite analysis of 6 markers including gp*60* was carried out to ensure that the selected isolates were genetically distinct. The results of the analysis revealed that all three of the selected isolates were genetically distinct at at least one locus (Table 3-4). BB and LH could be distinguished at 3 markers (MM5, MM19 and *gp60*), BB and OV at 2 markers (MM5 and MM19). LH and OV could only be distinguished at 1 marker (gp*60*).

Table 3-4: Alleles assigned to each isolate
Red text indicates markers at which the isolates differed.

Marker	Isolate BB	Isolate LH	Isolate OV
MM5	Allele 2	Allele 1	Allele 1
MM18	Allele 1	Allele 1	Allele 1
MM19	Allele 3	Allele 4	Allele 4
TP14	Allele 2	Allele 2	Allele 2
MS1	Allele 1	Allele 1	N/A
gp60	IIaA15G2R1	IIaA19G2R1	IIaA15G2R1

3.4.1.2 Faecal Consistency and Oocyst Shedding in Sampled Calves

These isolates presented different clinical signs in terms of oocysts shed and presence of diarrhoea in naturally infected calves (from personal observation and previous experience of *Cryptosporidium* infected calves on these two farms) (Table 3-5).

Table 3-5: Faecal consistancy and average oocysts counts associated with different *C. parvum* isolates in naturally infected calves

Isolate	Faecal Consistency	Average Oocyst Count (per ml)	Number of Samples Collected
BB	No diarrhoea	1.53×10^7	n=2
LH	Watery diarrhoea	1.72×10^6	n=6
OV	Slight diarrhoea	3.24×10^7	n=4

3.4.1.3 *C. parvum* isolates used in experimental trial

Isolates BB and LH were chosen as the ideal isolates to take forward to the trial as they differed in clinical manifestation as well as being genetically distinct at three loci. These isolates also came from calves on closed herd farms which decreased the likelihood that the infections were mixed. Isolate OV was excluded from the trial at this stage.

3.4.2 Production of inoculum

In total eight samples were obtained and were processed and the oocysts counted. The total numbers of oocysts obtained from each farm are shown in (Table 3-6). Samples containing high numbers of oocysts in a small volume of faeces were chosen for further processing to obtain oocysts for production of inoculum.

Table 3-6: Oocyst counts from faeces obtained from calves farms BB & LH

Sample	Type†	Volume (ml)	No. Oocysts	Total No. Oocysts
BB1*	3	194	1.84×10^9	3.75×10^9
BB2	3	90	1.91×10^9	
LH1	3	108	1.35×10^7	8.71×10^8
LH2*	5	73	6.02×10^8	
LH3*	5	65	6.91×10^7	
LH4	4	189	0	
LH5	3	294	1.10×10^8	
LH6*	5	152	7.60×10^7	

*these samples were processed by sucrose flotation to produce the inoculum for pre-amplification
† See Table 2-1

The oocysts obtained from LH2, LH3, LH6 were pooled as a single sample did not contain sufficient oocysts. Before producing inoculum the viability of the oocysts were purified by sucrose flotation (section 2.3.2.2) prior to an excystation assay which mimics the gut conditions and stimulates the oocysts to excyst, releasing sporozoites (section 2.3.3.1). A sporozoite:shell ratio of >2 and an excystation percentage of >85% is desirable (Katzer F, Pers Comm.) (Table 3-7).

Table 3-7: Sporozoite:Shell ratios and excystation percentages for each isolate

Isolate	Sporozoite:Shell ratio	Excystation %age
BB	2.8	97%
LH	2.1	88%

3.4.3 Pre-amplification of *Cryptosporidium parvum* isolates

Four neonatal lambs were successfully inoculated with two different *C. parvum* isolates (n=2 lambs per group/isolate) by oral infection using a syringe and 1×10^5 oocysts in PBS. All lambs showed clinical signs of disease (diarrhoea, inappetence) approximately three DPI (days post-infection) and oocyst shedding occurred in all animals (Figure 3-5).

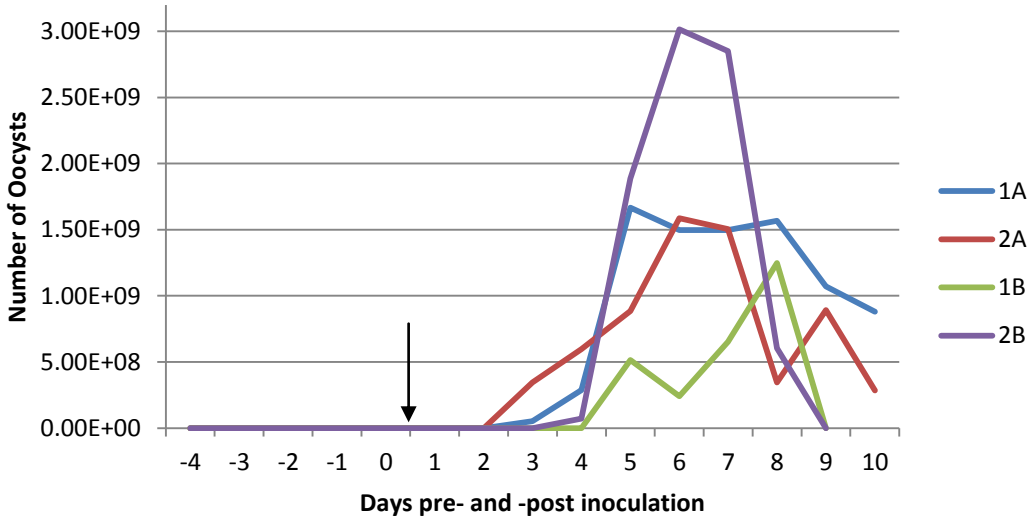


Figure 3-5: Shedding profile of lambs inoculated with BB (1A & 2A) and LH (1B & 2B) isolates.
Day of inoculation is indicated by the black arrow.

3.4.3.1 Sample Processing

Positive samples containing the highest number of oocysts in the smallest volume of faeces were processed by sucrose flotation to separate the oocysts from the faecal matter. The sucrose flotation step was repeated for samples from Group B as the faeces, in this case, was slightly thicker than ideal.

In total, 6.73×10^9 oocyst in 430 g faeces from Group A (BB) and 1.01×10^{10} oocysts in 596 g faeces from Group B (LH) were processed. From Group A, 2.36×10^9 oocysts were recovered (35% recovery) and from Group B, 5.5×10^8 oocysts were recovered (5% recovery).

A minimum of 4.2×10^7 oocysts of each isolate were required to inoculate all 36 lambs in the trial.

3.4.3.2 Excystation Assay

Cleaned oocysts were subjected to an excystation assay prior to each inoculation (<1 week & 6 weeks) (section 2.3.3.1) to determine the excystation percentage and sporozoite:shell ratio (Table 3-8).

Table 3-8: Sporozoite:Shell ratios and excystation percentages for inoculum used in experimental trial

Challenge	Isolate	Sporozoite:Shell ratio	Excystation %age
<1 week	BB	2.21	95%
	LH	2.94	92%
6 weeks	BB	2.04	74%
	LH	1.31	82%

The excystation percentages and sporozoite:shell ratios were slightly lower than ideal (>2 sporozoite:shell ratio/>85% excystation) for the challenge at 6 weeks of age.

3.4.4 Experimental Animals – clinical observations

All lambs drank well even following infection, some animals showed mild signs of abdominal pain (lying down after feeding) one or two days after infection but they did not stop feeding. Feed intake between groups was similar (**Error! Reference source not found.**).

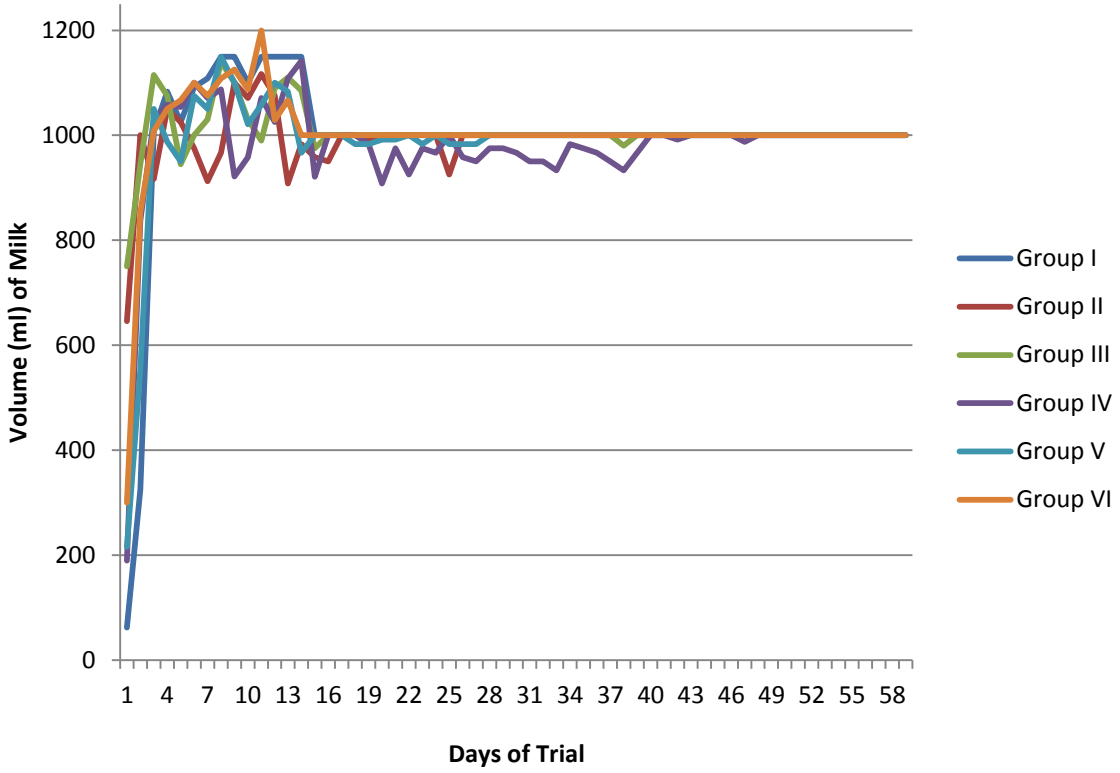


Figure 3-6: Average feed intake of each group.
Lambs did not go off their feed following infection with *C. parvum.*

3.4.4.1 Age-related Susceptibility to Disease

To determine whether animals become less susceptible to disease as their age increases two groups (Group I and Group II) were infected with *C. parvum* at 6 weeks of age and the disease outcome and oocyst shedding was compared with lambs which were infected at <1 week of age (Group III, Group IV, Group V and Group VI). Lambs infected at 6 weeks of age were given with a higher dose (5×10^6) than animals infected at <1 week of age (1×10^6) (Figure 3-1)..

Isolate BB

Animals which were infected with BB for the first time at 6 weeks of age (Group I) showed milder clinical disease (number of diarrhoeic samples and duration of diarrhoea) compared with animals infected with this isolate for the first time at <1 week of age (Group III & Group IV). Overall, following primary infection at 6 weeks of age only one sample collected from one animal in Group I (BB wk6) was

scored as diarrhoeic compared with 12 samples from four animals in Group III and 32 samples from six animals in Group IV (BB wk1). (Figure 3-7).

Isolate LH

Animals which were infected with isolate LH for the first time at 6 weeks of age (Group II)showed milder clinical disease compared with animals infected for the first time at <1 week of age (Group V & Group VI).Overall, following primary infection at 6 weeks of age five samples collected from one animal in Group II were scored as diarrhoeic. In contrast, 23 samples collected from six animals in Group V and 55 samples from six animals in Group VI were scored as diarrhoeic. (Figure 3-7).

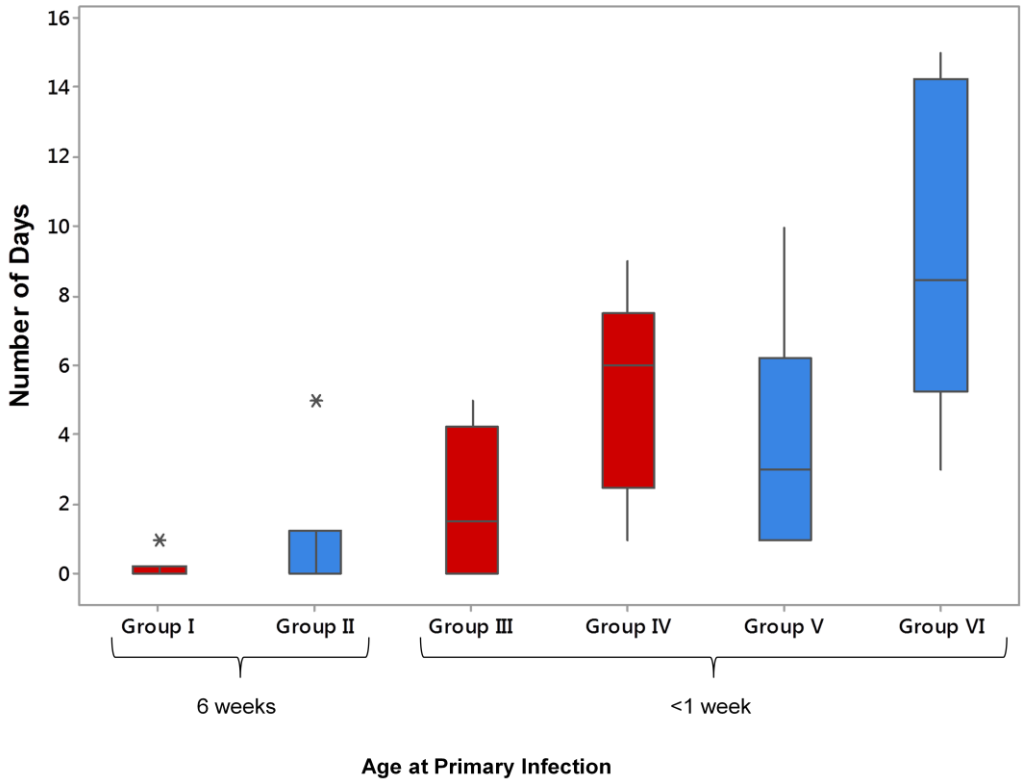


Figure 3-7: Number of days lambs produced a diarrhoeic sample following primary-infection at <1 week (Group III, IV, V & VI) and 6 weeks (Group I & II) of age.
Lambs infected at 6 weeks of age produced fewer diarrhoeic samples than lambs infected at 6 weeks of age. Red bars show primary infection with BB isolate and blue bars show primary infection with LH isolate. (* indicates outliers, the horizontal line the median and the vertical line indicates that the median is zero).

Animals challenged at 6 weeks (Group I & II) had a shorter duration of diarrhoea than animals challenged at <1 week of age (Group III, IV, V & VI) and produced fewer diarrhoeic samples (P<0.001).

3.4.4.2 Difference between isolates

To compare the pathogenicity of the two isolates one age-related susceptibility, one homologous and one heterologous challenge per isolate was carried out. Results show that the isolate used in the primary infection at <1 week of age does not significantly affect the probability of diarrhoea during the first three weeks post-infection . There was no significant effect of isolate on diarrhoea following challenge at 6 weeks of age.

Isolate had a significant effect on the interval to onset of diarrhoea (P < 0.05, Table 3-9a and Figure 3-8) such that isolate LH resulted in earlier onset diarrhoea following primary infection. There was no effect of isolate on days to diarrhoea after the second challenge (P= 0.434, Table 3-9b and Figure 3-8).

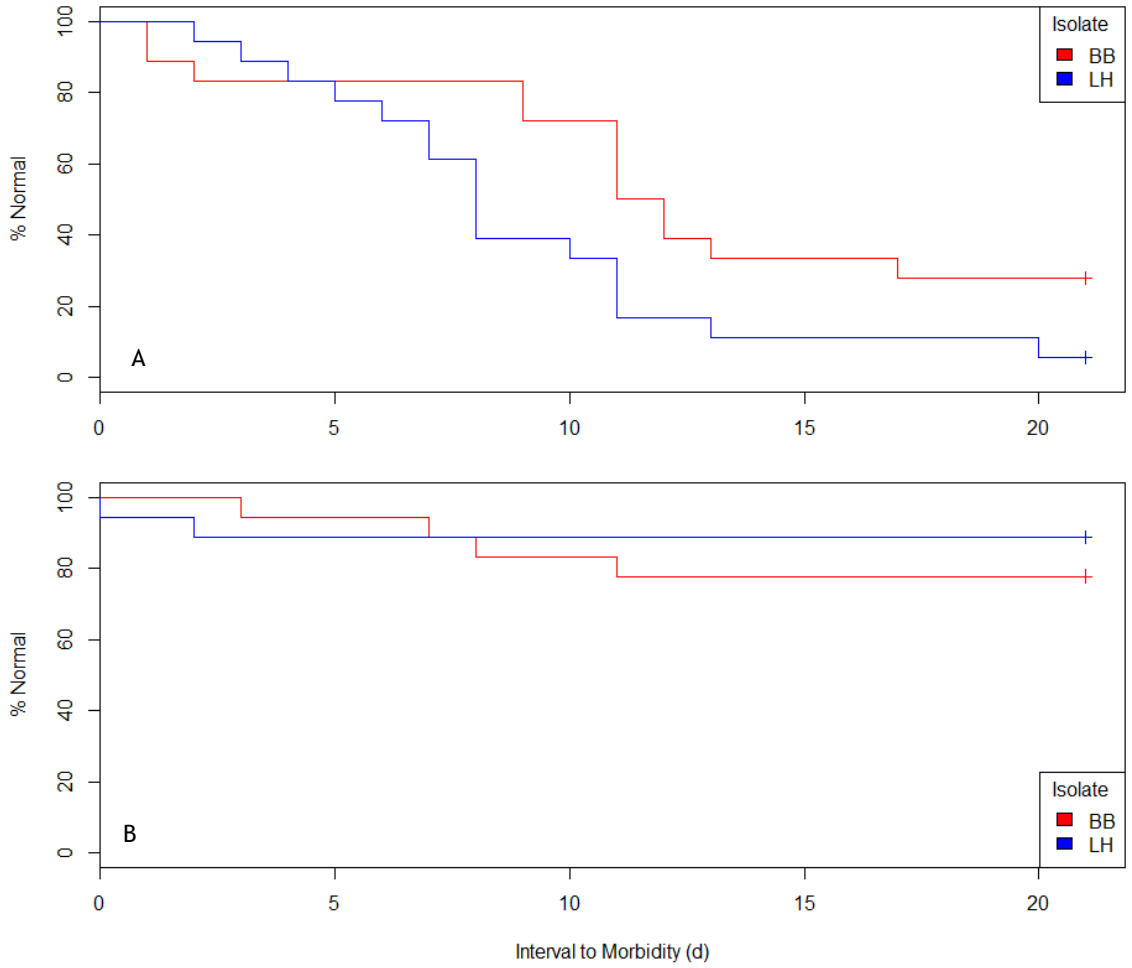


Figure 3-8: Time to onset of diarrhoea following primary challenge at <1 week (A) and 6 weeks (B) of age.
Lambs challenged with isolate LH at <1 week of age began scouring sooner than animals infected with isolate BB. There was no difference in time to onset of diarrhoea following challenge at 6 weeks of age.

Table 3-9a: Log-rank test on Kaplan-Meier survival curves for onset of diarrhoea after challenge at <1 week of age.

Isolate 1	N	Observed(O)	Expected(E)	(O-E)^2/E	(O-E)^2/V
BB	18	13	18.5	1.61	4.91
LH	18	17	11.5	2.58	4.91
Chisq= 4.9 on 1 degrees of freedom, p= 0.0266					

Table 3-9b: Log-rank test on Kaplan-Meier survival curves for onset of diarrhoea after challenge at 6 weeks of age.

Isolate 1	N	Observed(O)	Expected(E)	(O-E)^2/E	(O-E)^2/V
BB	18	4	3.04	0.301	0.612
LH	18	2	2.96	0.310	0.612
Chisq= 0.6 on 1 degrees of freedom, p= 0.434					

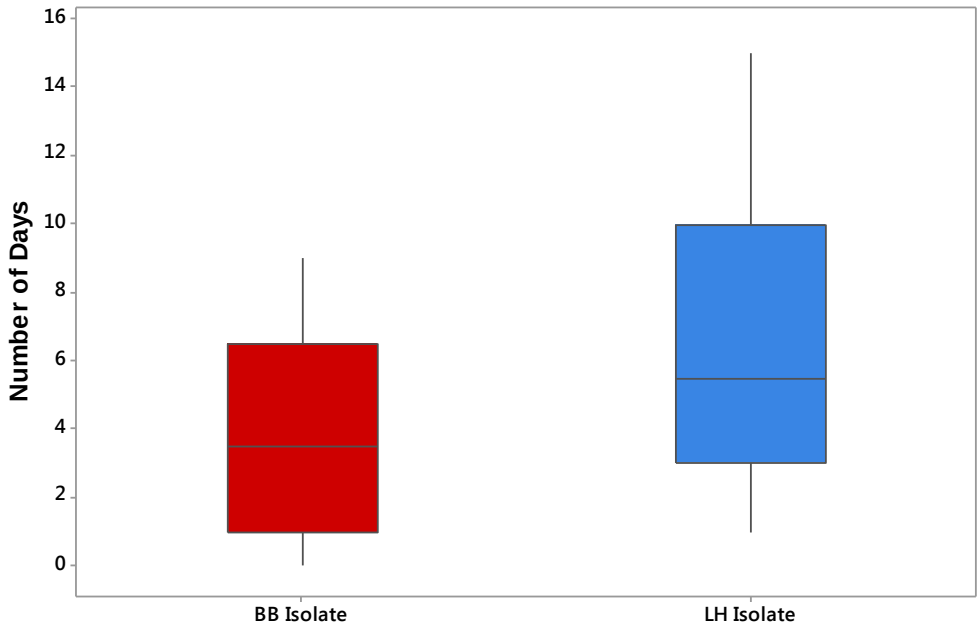


Figure 3-9: Number of days lambs infected with BB (red) or LH (blue) isolate produced a diarrhoeic sample in the 3 weeks post-primary infection at <1 week of age.

3.4.4.3 Previous exposure (homologous and heterologous challenge)

To determine if previous exposure to *C. parvum* provides protection against a secondary challenge later in life two sequential challenges were carried out to test each isolate in a homologous and heterologous challenge.

All animals which received a primary infection or secondary challenge at 6 week of age showed a reduction in clinical disease compared with animals which received a primary infection at <1 week of age. Previous exposure did not provide significant protection (P>0.99) against re-infection and the occurrence of clinical disease. The effect which age had on the occurrence of diarrhoea masked any effect that previous exposure may have had. There was no significant difference in the occurrence (P>0.99) or days of diarrhoea (p=0.378) between groups with previous exposure (Group III, IV, V & V) and groups which had no previous exposure (Group I & II) (Figure 3-10 and Table 3-10).

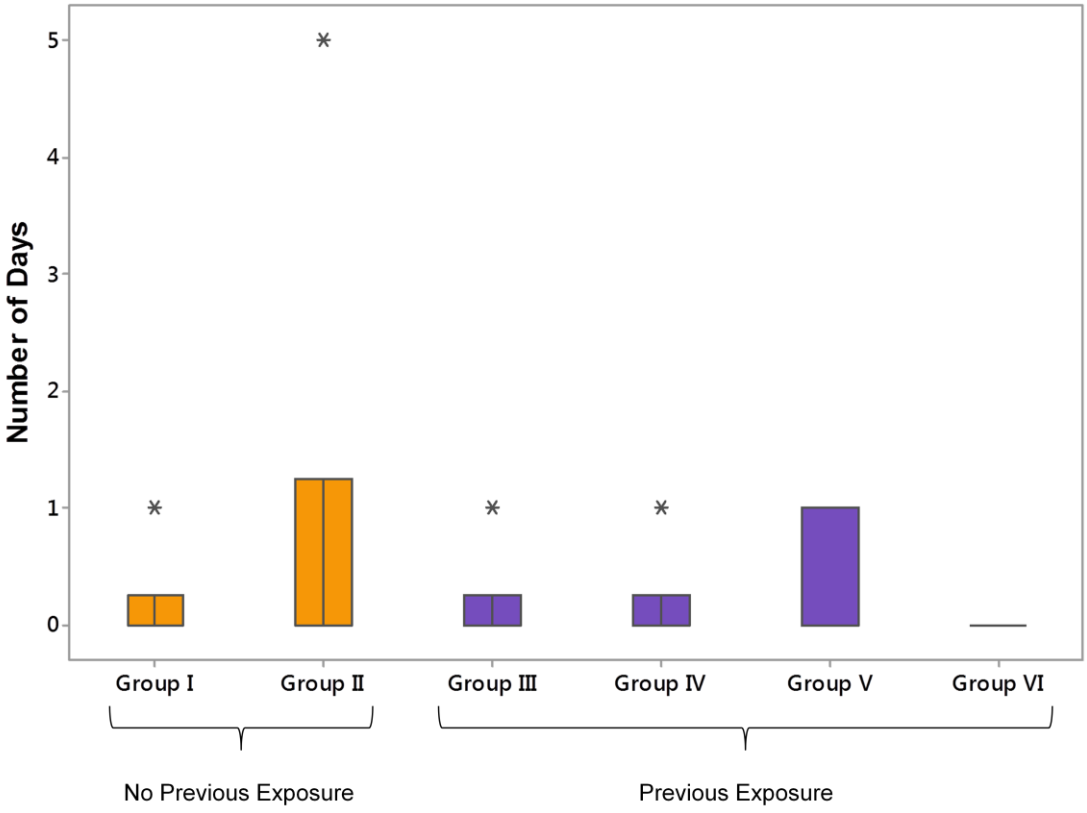


Figure 3-10: Occurrences of diarrhoea following challenge at 6 weeks
There was no difference in the occurrence or days of diarrhoea following challenge at 6 weeks in lambs receiving (purple boxes) or not receiving (orange boxes) a primary infection. (* indicates outliers, the horizontal line the median and the vertical line indicates that the median is zero).

There was no difference (P=0.571) in clinical disease caused by a homologous or heterologous challenge (Table 3-10), again, any effect of the type of challenge (homologous or heterologous) may be masked by the effect of age.

Table 3-10: Multivariate logistic regression model for analysis of factors associated with occurrence of diarrhoea in wk1 – wk4 and wk6 – wk8.

Outcome Variable	Explanatory Variable		1	0	Odds Ratio	95% CI	P-value
D1	**Isolate**						**0.086**
		BB	13	5	1		
		LH	17	1	*	0.00 - *	
	Group						**0.0837**
		I	3	3	1		
		II	5	1	5.00	0.55 – 45.39	
		III	4	2	2.00	0.31 – 13.06	
		IV	6	0	0	0.00 - *	
		V	6	0	0	0.00 - *	
		VI	6	0	0	0.00 - *	
D2	**Isolate**						**1**
		BB	4	14	1		
		LH	2	16	1	0.17 – 5.77	
	Group						**0.0977**
		I	1	5	1		
		II	1	5	1	0.05 – 20.83	
		III	1	5	1	0.05 – 20.83	
		IV	1	5	1	0.05 – 20.83	
		V	2	4	2.5	0.16 – 38.60	
		VI	0	6	0	0.00 - *	
	Type						**0.571**
		*	2	10	1		
		Homologous	1	11	1.67	0.22 – 12.37	
		Heterologous	3	9	0.45	0.04 – 5.81	
	Challenge						**1**
		Primary	1	10	1		
		Secondary	4	20	1	0.16 – 6.42	

3.4.5 Experimental Animals – oocyst shedding

Due to time constraints it was not possible to carry out oocyst counts for all groups, instead oocyst counts were carried out for four of the six groups. Oocyst

counts were carried out on samples from animals in Group I & II (age-related susceptibility to disease), Group III (homologous challenge with BB) and Group V (heterologous challenge).

3.4.5.1 Group I

Oocysts counts were carried out on 167 (52.35%) samples from six lambs in Group I. One sample from each lamb was counted each week for the first 6 weeks to ensure that the animal remained *Cryptosporidium*-free for this period. Following primary challenge every sample produced by each lamb was processed and counted. The number of samples processed from each lamb is summarised in Table 3-11.

Table 3-11: Samples collected and processed from each lamb in Group I

Lamb	Samples Collected	Samples Processed and Counted	Percentage (%) of Samples Processed
A	59	30	50.84
B	60	22	36.60
C	46	26	52.38
D	54	29	53.70
E	57	32	56.14
F	43	28	65.11

None of the lambs showed any signs of *Cryptosporidium* infection in the first 6 weeks of life and all samples tested were negative for oocysts. Following primary challenge at 6 weeks lambs started to shed oocysts at 1-2 DPI, shedding continued for 12-16 days before tailing off. Peaks in oocyst shedding occurred at 2, 4, 8, 11 & 15 DPI in individual animals (Figure 3-11). Oocyst shedding between lambs differed significantly (P=0.037) with one lamb (C) shedding higher numbers of oocysts than other animals (Figure 3-12).

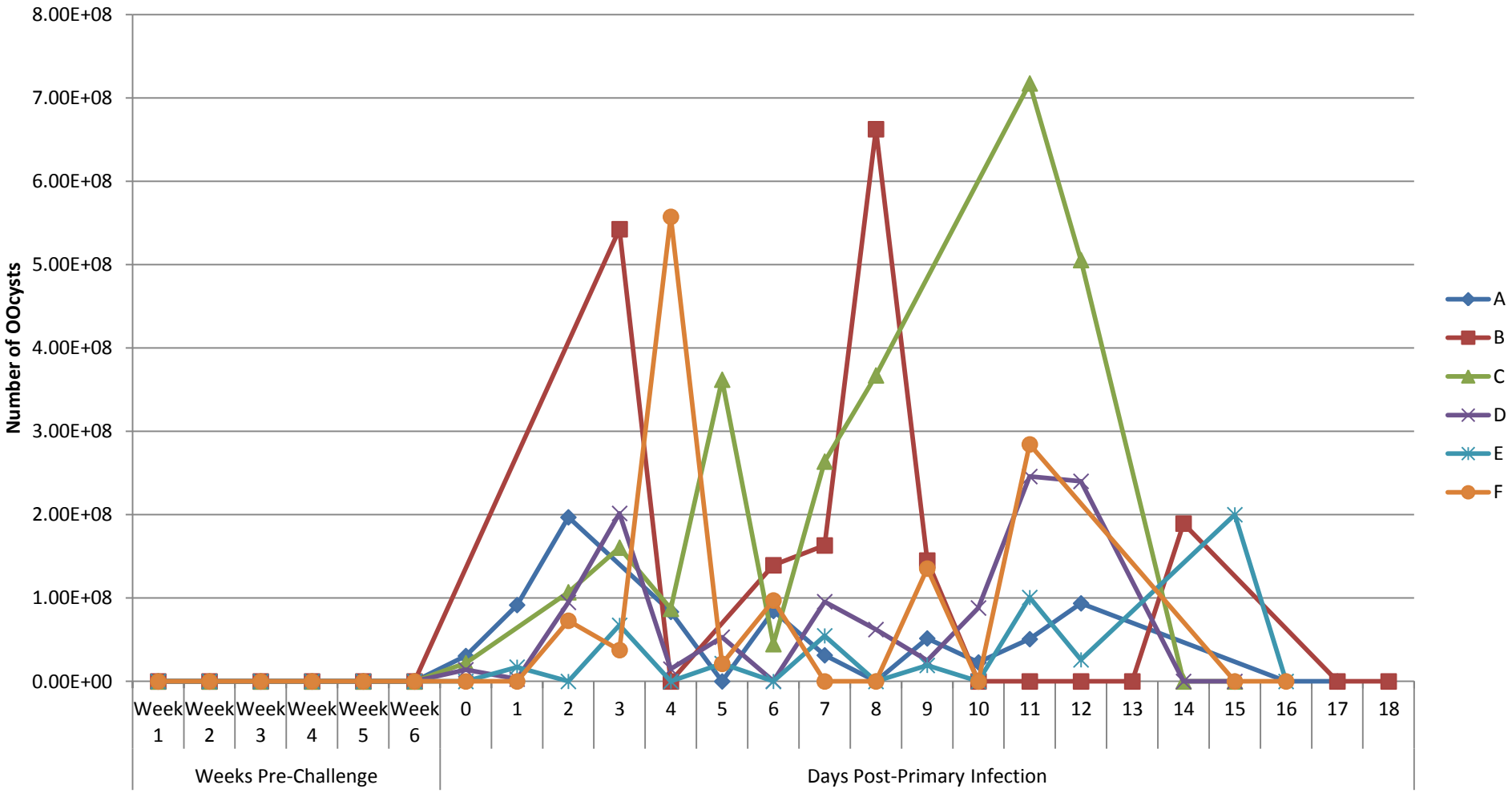


Figure 3-11: Oocyst counts from lambs in Group I

Shedding profiles from lambs in Group I following primary challenge at 6 weeks of age with 5×10^6 *C. parvum* oocysts isolate BB. Lambs began shedding oocysts very soon after inoculation with peaks in oocyst shedding at 2, 4, 8, 11 & 15 DPI in individual animals. Shedding began to tail off at 14 DPI. It was not possible to collect a sample from each animal on each day, days on which samples were collected are indicated by a data point.

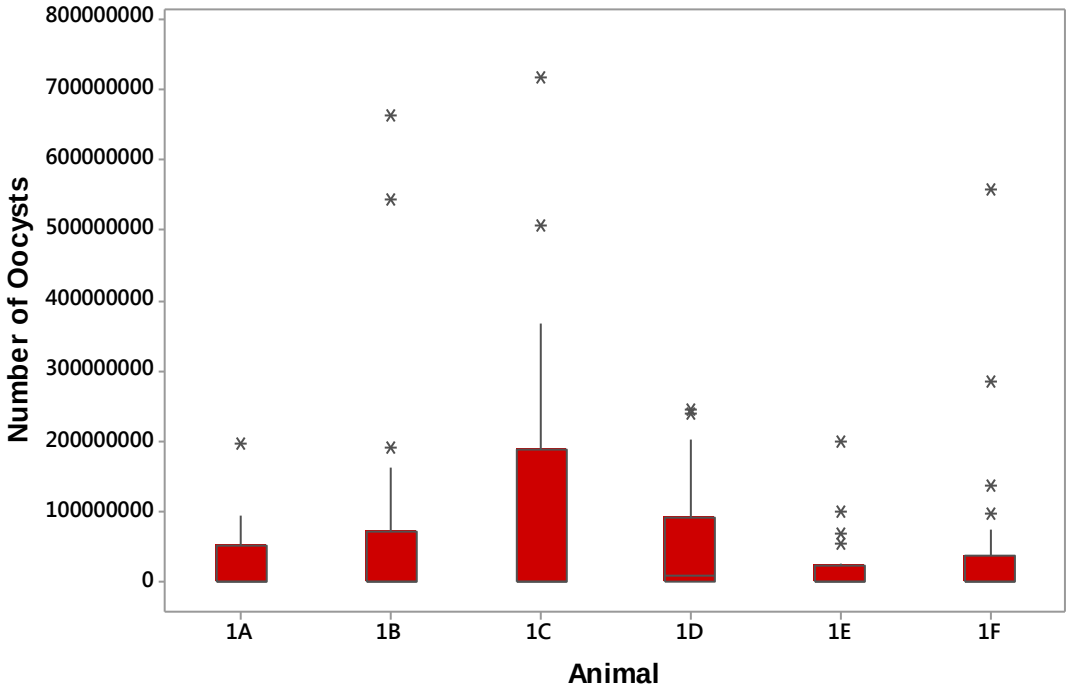


Figure 3-12: Total number of oocysts shed by individual lambs in Group I (BB wk6)
Oocyst shedding between individual animals within the same group varied with some animals shedding significantly more oocysts than others. (* indicates outliers).

3.4.6 Group II

Oocysts counts were carried out on 150 (48.85%) samples from six lambs in Group II. At least one sample from each lamb was counted each week for the first 6 weeks to ensure that the animal remained *Cryptosporidium*-free for this period. Following primary challenge every sample produced by each lamb was processed and counted. The number of samples processed from each lamb is summarised in (Table 3-12).

Table 3-12: Samples collected and processed from each lamb in Group II

Lamb	Samples Collected	Samples Processed and Counted	Percentage (%) of Samples Processed
A	50	30	60.00
B	63	28	44.44
C	59	26	44.06
D	39	21	53.84
E	45	19	42.22
F	51	26	50.98

None of the lambs showed any signs of *Cryptosporidium* infection in the first 6 weeks of life and all samples tested (n=52) were negative for oocysts. Following primary challenge at 6 weeks lambs started to shed oocysts at 1-2 DPI, shedding continued for 11 days before tailing off. Peaks in oocyst shedding occurred at 3, 5, 8 & 10 DPI in individual animals (Figure 3-13). Oocyst shedding between lambs differed but the difference in shedding was not significant (Figure 3-14).

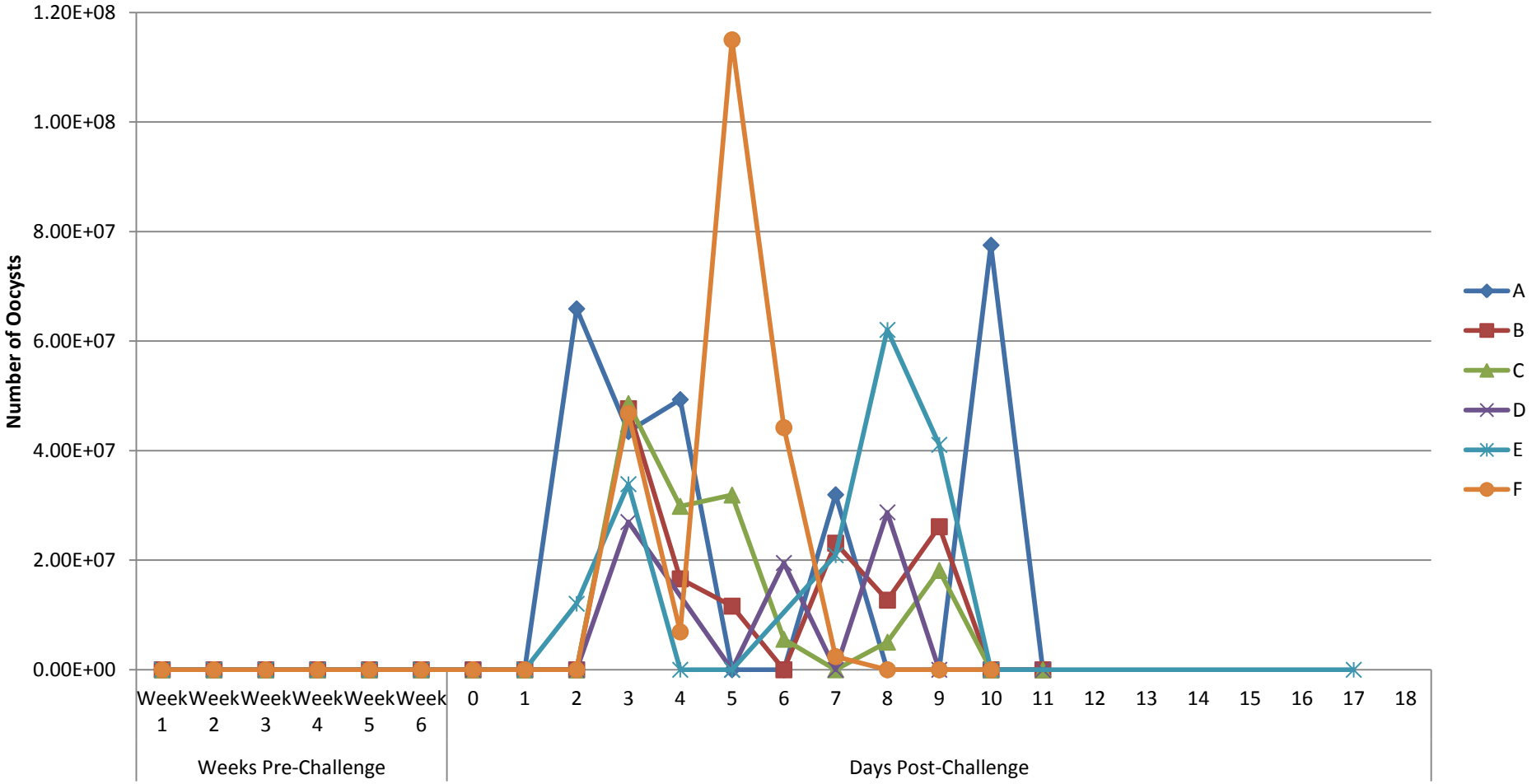


Figure 3-13: Oocyst counts from lambs in Group II
Shedding profiles from lambs in Group II following primary challenge at 6 weeks of age with 5×10^6 *C. parvum* oocysts isolate LH. Lambs began shedding oocysts very soon after inoculation with a peak in shedding at approximately 3 DPI. Shedding began to tail off at 11 DPI. It was not possible to collect a sample from each animal on each day, days on which samples were collected are indicated by a data point.

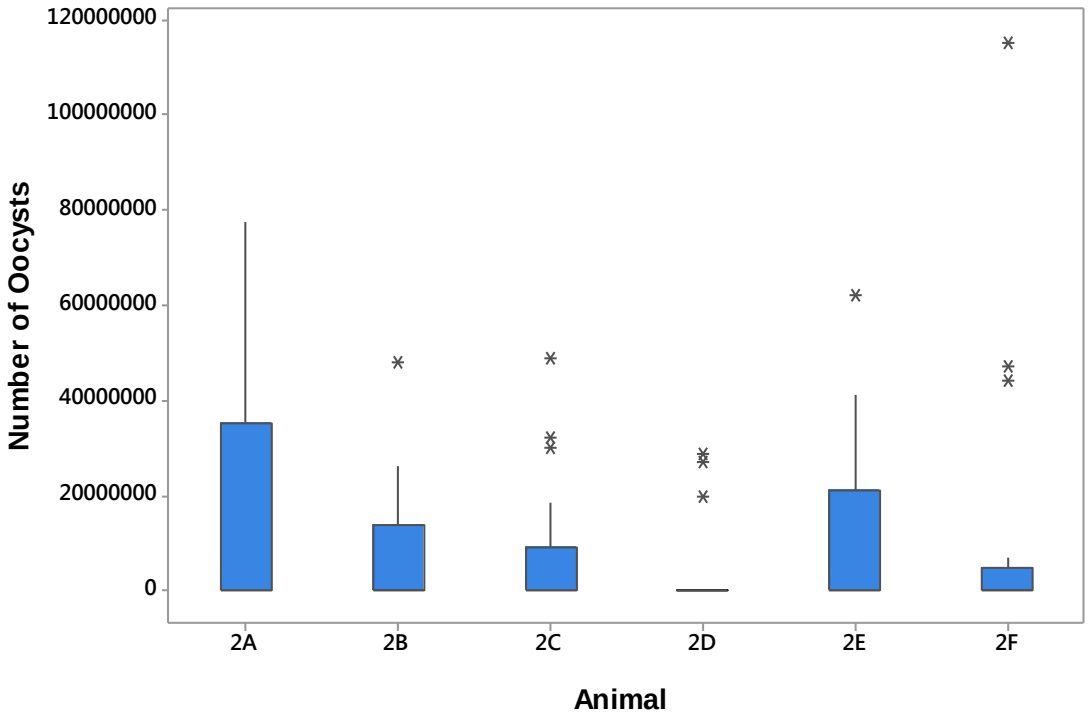


Figure 3-14: Total number of oocysts shed by individual lambs in Group II.
Oocyst shedding between lambs in the same group varied but not significantly. (* indicates outliers).

3.4.7 Group III

Oocysts counts were carried out on 335 (73.62%) samples from six lambs in Group III. Following primary infection (BB) every sample produced by each lamb was processed and counted for 3-4 weeks then 1-2 samples per week for two weeks and then every sample following secondary challenge (BB) for a further 19 days. The number of samples processed from each lamb is summarised in Table 3-13.

Table 3-13: Samples collected and processed from each lamb in Group III

Lamb	Samples Collected	Samples Processed and Counted	Percentage (%) of Samples Processed
A	65	51	78.46
B	73	58	79.45
C	83	67	80.72
D	82	69	84.14
E	74	60	81.08
F	68	50	73.53

None of the lambs showed any signs of *Cryptosporidium* infection in the days prior to primary challenge and all samples tested (n=26) were negative for *Cryptosporidium* oocysts. Following primary infection lambs began to shed oocysts at 14 DPI, shedding continued until 26-28 DPI when oocysts shedding tailed off for the majority of animals. Peaks in oocysts shedding occurred at 15, 18, 21 and 23 DPI in five of the six animals. Within this group animal F consistently shed higher numbers of oocysts than the others with peaks in shedding occurring at Days 24 and 31 PI. By 27 DPI oocyst shedding had decreased to $<2.5 \times 10^7$ in most of the animals (Figure 3-15 & Figure 3-16).

Animals in this group were re-challenged with the same isolate (BB) at 39 days of age, the dose of the secondary challenge was higher than the primary infection (primary infection 1×10^6 and secondary challenge 5×10^6).

Following secondary challenge animals began shedding oocysts at 2-4 DPI and continued to shed oocysts for 10–17 days. Peaks in shedding occurred at 5,7 and 11 DPI in the majority of animals, again animal F shed higher numbers of oocysts

than the others and in this animal peaks in oocyst shedding occurred at 4, 6, 9, 12, 14 and 17 DPI.

All animals shed more oocysts following secondary challenge than following primary infection with the same isolate. In total the lambs in this group shed 1.80×10^{10} oocysts, 4.97×10^{9} following the primary infection and 1.31×10^{10} following the secondary challenge.

There was a significant difference ($P<0.001$) between the number of oocysts each lamb shed with some lambs, particularly animal F shedding higher numbers of oocysts than the others. This animal shed ~5.00×10^{9} oocysts more than the other animals in this group following secondary challenge (Figure 3-17).

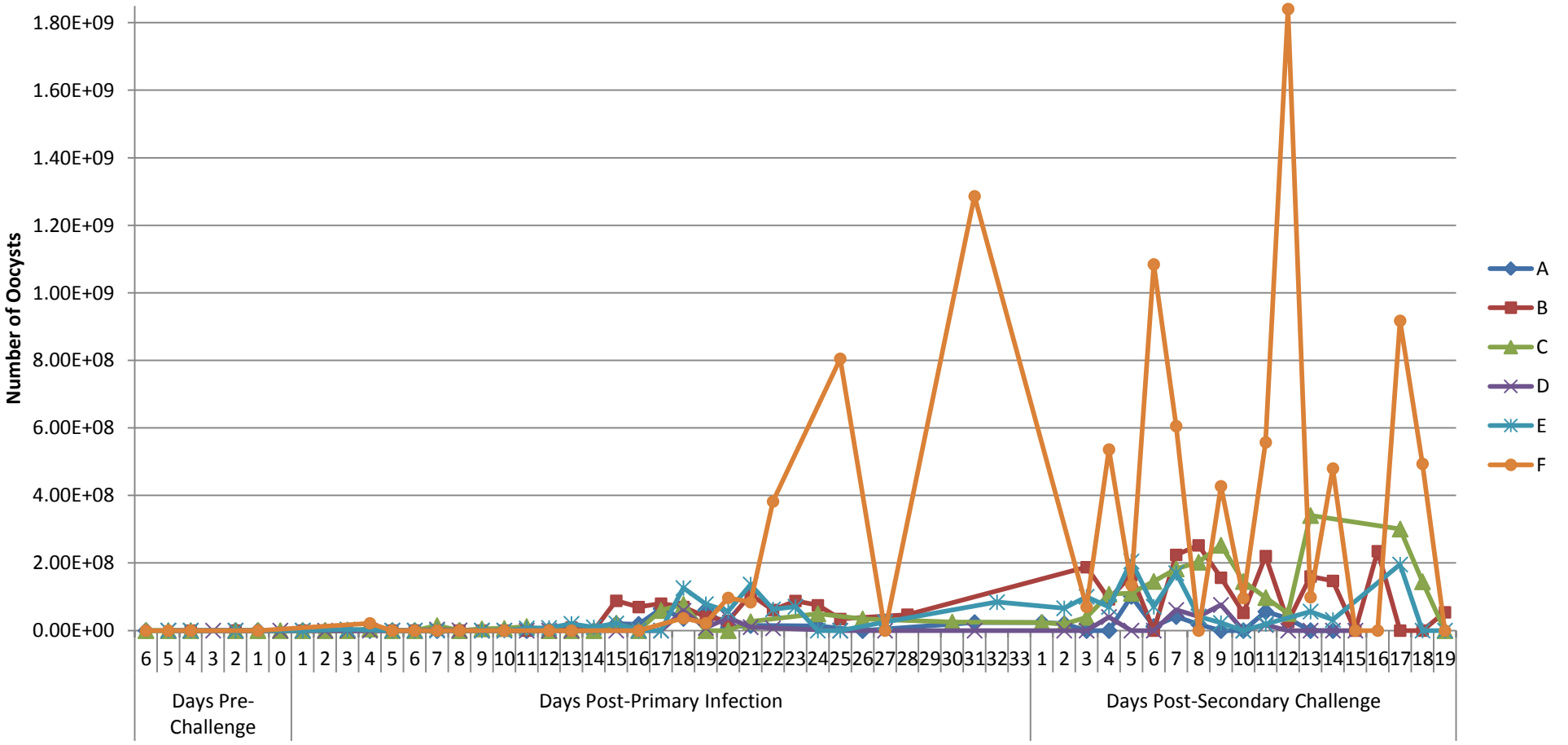


Figure 3-15: Oocyst counts from animals in Group III
Shedding profiles from lambs in Group III following primary infection at <1 week of age with 1×10^6 *C. parvum* oocysts isolate BB and challenge at 6 weeks of age with 5×10^6 *C. parvum* oocysts isolate BB. Shedding began to tail off at 14 DPI. It was not possible to collect a sample from each animal on each day, days on which samples were collected are indicated by a data point.

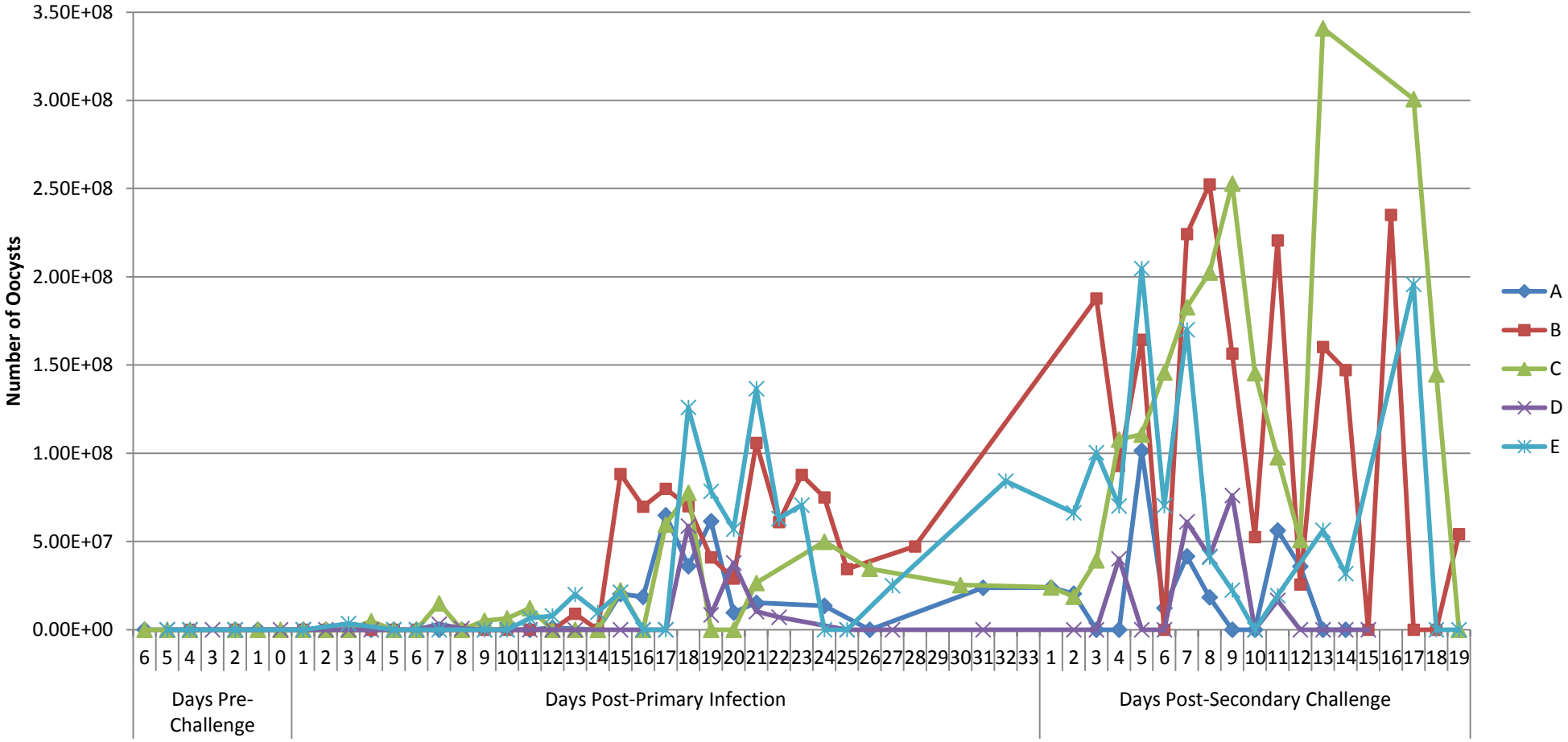


Figure 3-16: Oocyst counts from animals in Group III excluding animal F
Shedding profiles from lambs in Group III following primary challenge at <1 week of age with 1×10^6 *C. parvum* oocysts isolate BB and re-challenge at 6 weeks of age with 5×10^6 *C. parvum* oocysts isolate BB. It was not possible to collect a sample from each animal on each day, days on which samples were collected are indicated by a data point. Animal F has been excluded from this graph in order to see the shedding profile of the other 5 lambs more clearly. Animal F shed more oocysts than the other lambs in the study.

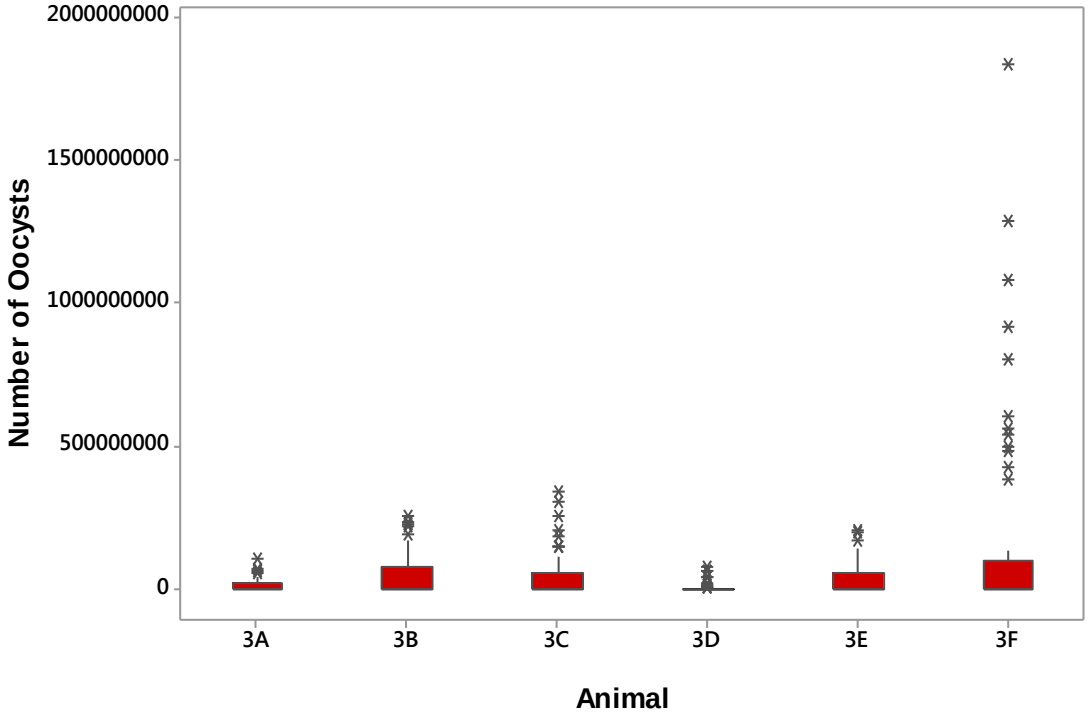


Figure 3-17: Total number of oocysts shed by individual lambs in Group III
Oocyst shedding between individual animals within the same group varied with some animals shedding significantly more oocysts than others. (* indicates outliers).

3.4.8 Group V

Oocysts counts were carried out on 275 (75.55%) samples from 6 lambs in Group V. Following primary infection (LH) every sample produced by each lamb was processed and counted for 3-4 weeks then 1-2 sample per week for two weeks and then every sample following secondary challenge (BB) for a further 15 days. The number of samples processed from each lamb is summarised in Table 3-14.

Table 3-14: Samples collected and processed from each lamb in Group V

Lamb	Samples Collected	Samples Processed and Counted	Percentage (%) of Samples Processed
A	64	43	67.19
B	67	53	79.10
C	67	48	71.64
D	69	51	73.91
E	51	38	74.51
F	46	42	91.30

None of the lambs showed any signs of *Cryptosporidium* infection in the days prior to primary challenge and all samples tested (n=26) were negative for *Cryptosporidium* oocysts. Following primary infection lambs began to shed oocysts at 11-18 DPI, shedding within animals in this group did not tail off as in other groups. In the majority of animals oocyst shedding continued at $<1.00 \times 10^8$ until they were re-challenged at 38 days of age. Peaks in oocysts shedding occurred at 16, 21 and 30 DPI in most of the lambs although oocysts shedding within animals in Group V was more sporadic than in other groups.

Animals in this group were challenged with a different *C. parvum* isolate (BB) at 38 days of age, the dose of the secondary challenge was higher than the primary infection (primary infection 1×10^6 and secondary challenge 5×10^6).

Following secondary challenge animals continued shedding oocysts and all animals showed an increase in the number of oocysts shed compared with the previous challenge. Following secondary challenge oocyst shedding continued for 10–15 days. Peaks in shedding occurred at 2,5 and 11 DPI in the majority of animals (Figure 3-18 & Figure 3-19) with animals E & F shedding significantly ($P<0.001$) higher numbers of oocysts than the others (Figure 3-20).

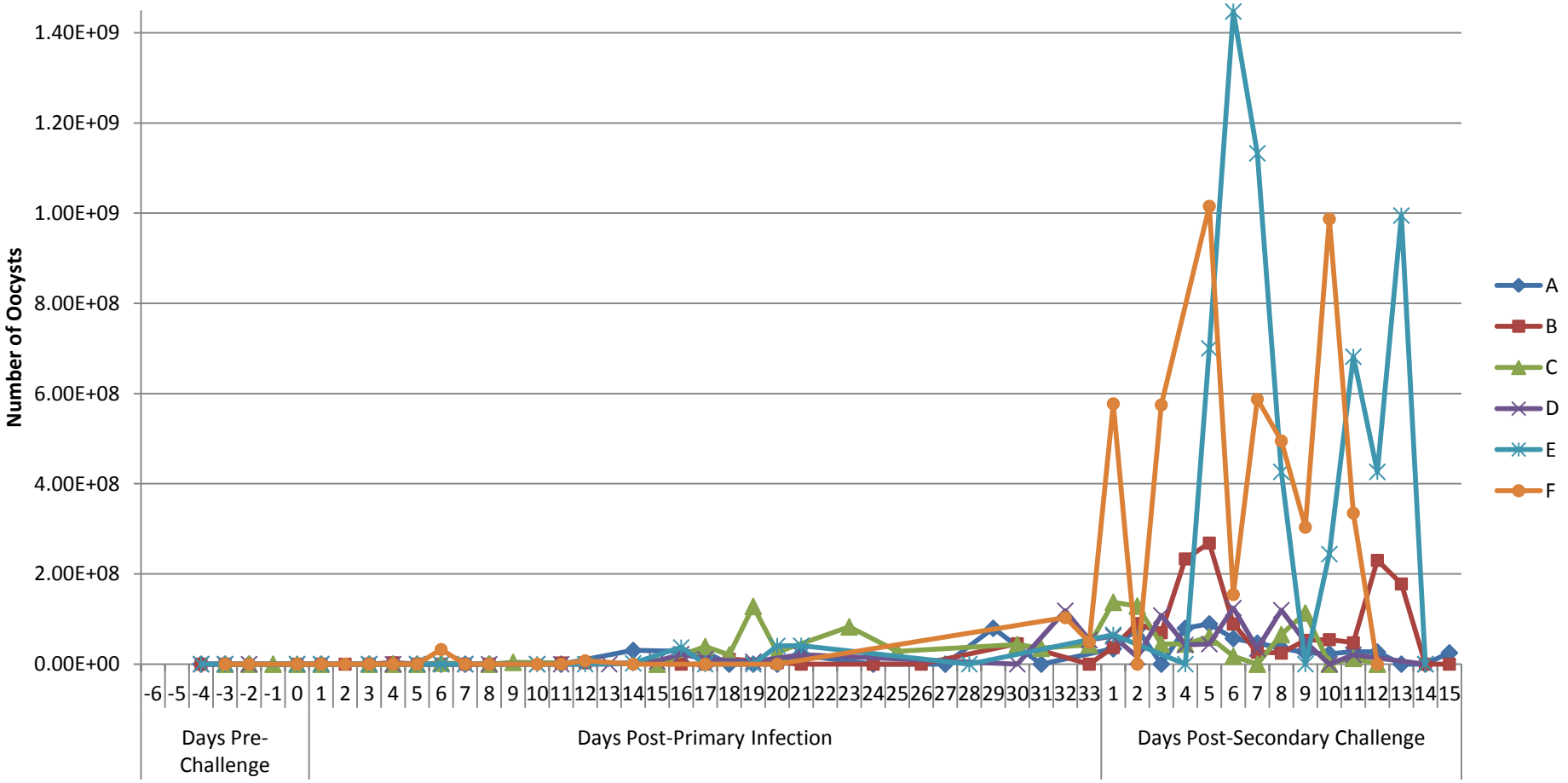


Figure 3-18: Oocyst counts from Group V
Shedding profiles from lambs in Group V following primary challenge at <1 week of age with 1×10^6 *C. parvum* oocysts isolate LH and re-challenge at 6 weeks of age with 5×10^6 *C. parvum* oocysts isolate BB. It was not possible to collect a sample from each animal on each day, days on which samples were collected are indicated by a data point.

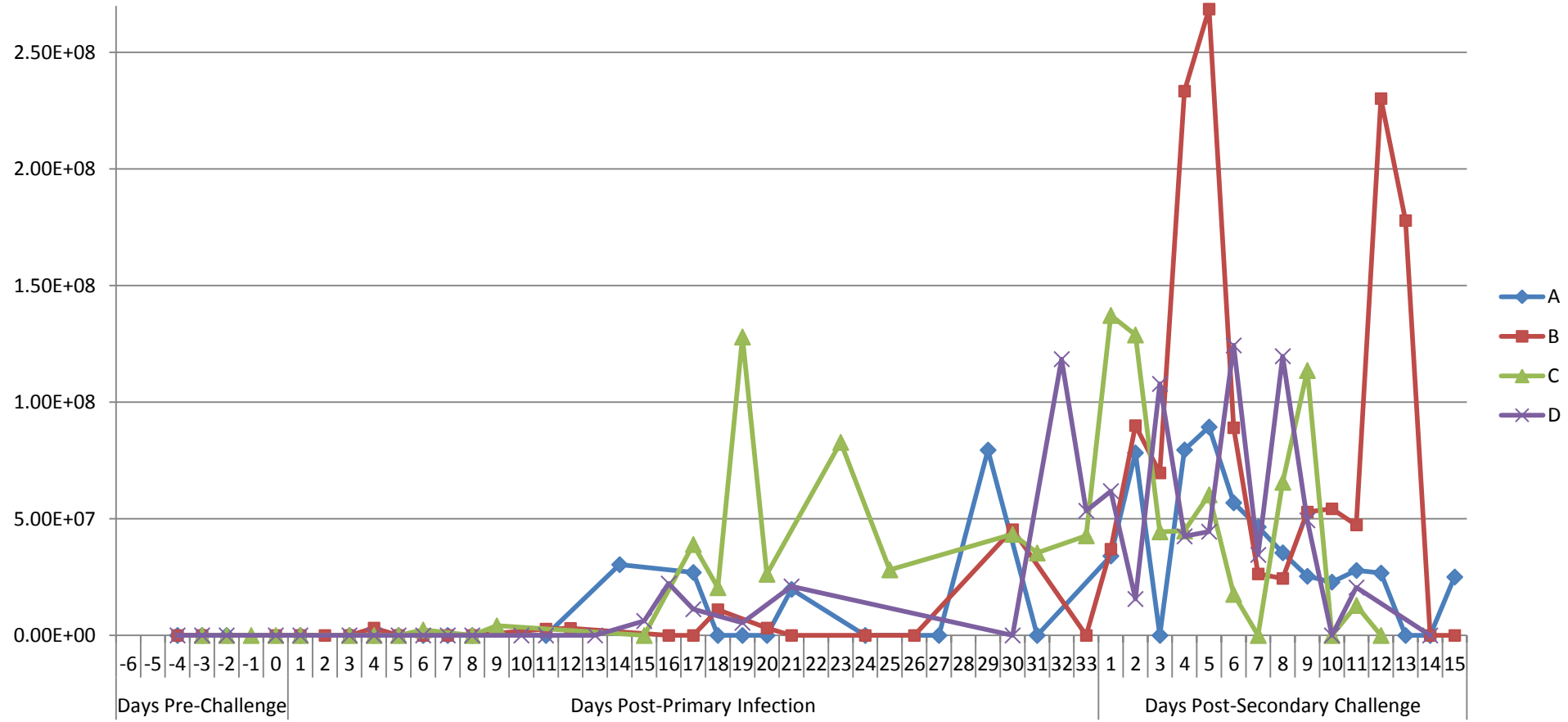


Figure 3-19: Oocyst counts from Group V excluding animals E & F
Shedding profiles from lambs in Group V following primary challenge at <1 week of age with 1 × 10^6 *C. parvum* oocysts isolate LH and re-challenge at 6 weeks of age with 5 × 10^6 *C. parvum* oocysts isolate BB. It was not possible to collect a sample from each animal on each day, days on which samples were collected are indicated by a data point. Animals E & F have been excluded from this graph in order to see the shedding profile of the other 4 lambs more clearly. Animals E & F shed more oocysts than the other lambs in the study.

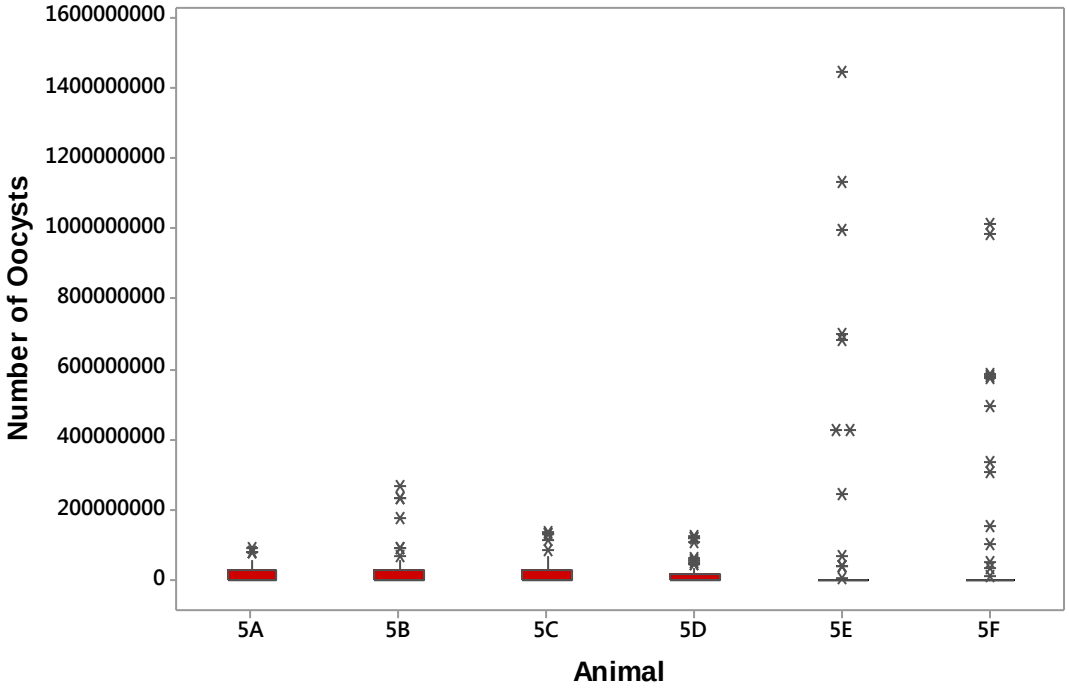


Figure 3-20: Total number of oocysts shed by individual lambs in Group V
Oocyst shedding between animals in group V varied significantly with lambs E and F shedding more oocysts than the other lambs. (* indicates outliers).

3.5 Effect of age and infective dose on shedding

When comparing the patterns of oocyst shedding between the groups, animals which were infected at <1 week of age with a low dose (1×10^6) began shedding oocysts after approximately 14 DPI whereas animals which were infected for the first time at 6 weeks of age with a higher dose (5×10^6) began shedding after a much shorter period of 1-2 DPI. Animals re-challenged with a higher dose (5×10^6) at 6 weeks of age (Group III & V) also began shedding oocysts sooner at 1-2 DPI. There was no significant difference (P=0.457) in number of oocysts shed between animals infected at <1 week of age and animals infected at 6 weeks of age.

3.5.1 Variation between isolates

Following primary infection at <1 week of age (Group III & Group V) there was no significant difference in number of oocysts shed and the isolate which the lambs were infected with (P=0.059). Following primary infection (Group I & Group II)

and secondary challenge (Group III & Group V) at 6 weeks of age infection with BB isolate was significantly associated with higher oocyst output ($P<0.001$).

3.5.2 Previous Exposure

Animals which had been previously exposed to *C. parvum* (Group III) & Group V) shed more oocysts following secondary challenge at 6 weeks of age than naïve animals (Group I & Group II) which received a primary infection at the same age ($P=0.02$)

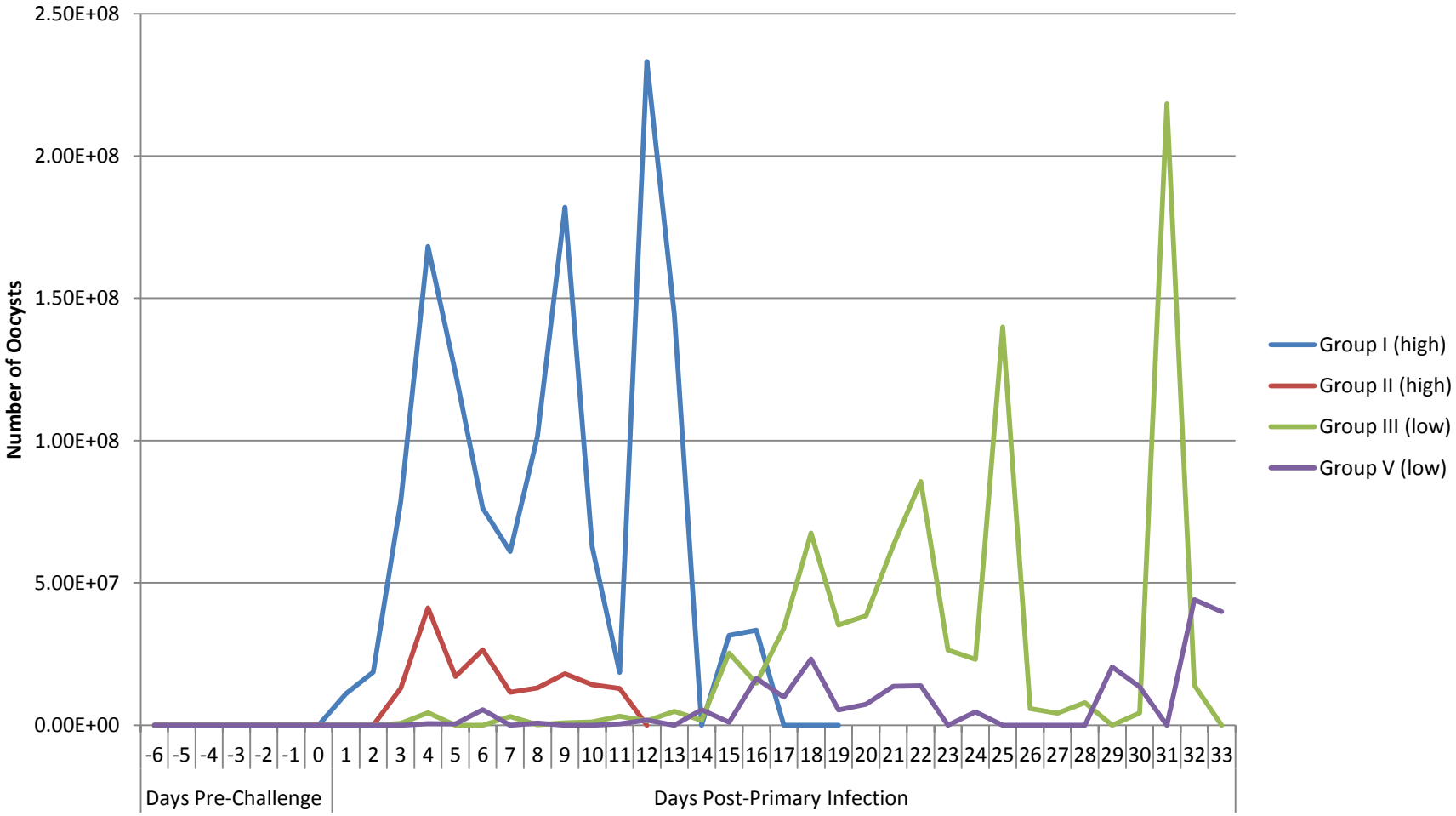


Figure 3-21 Average oocysts counts (Group I, II, III & V) following primary infection.
Lambs receiving a high (5×10^6) dose at primary infection shed more oocysts and had a shorter prepatent period than lambs receiving a low (1×10^6) dose.

3.6 Discussion

Infection with *Cryptosporidium* is very common in young ruminant livestock (de Graaf et al., 1999). As reported, in this thesis (Chapter 5 & Chapter 6) and other work, animals may become infected with *Cryptosporidium* at a very early age (Ortega-Mora et al., 1999; Tzipori et al., 1983) indicating that they have been exposed to the parasite almost immediately at birth. As a result it is difficult to examine the development of resistance to disease in a field setting. In this experiment the susceptibility of lambs to disease caused by two distinct *C. parvum* isolates was tested by a primary infection at <1 week and a homologous or heterologous challenge at 6 weeks of age as well as testing the susceptibility of lambs kept in isolation from the parasite and then experimentally challenged at 6 weeks of age.

Although the isolates used in this trial were originally derived from naturally infected calves it was decided to carry out the trial using lambs. From previous and ongoing work at the Moredun Research Institute it is known that lambs are susceptible to infection with *C. parvum* and exhibit similar clinical signs and patterns of oocyst shedding. Lambs are smaller and easier to handle than calves especially once they grow to 8 weeks of age. In addition, total faecal output from each individual animal was collected for the duration of the trial. Total faecal output from a calf infected with *C. parvum* could be as much as 3 kg per day compared to 600 g from a lamb (Bartley, Pers. Comm.). The results of this study suggest that lambs can be used as a model for disease outcome and shedding in calves as the clinical disease and shedding patterns of experimentally infected lambs, in general, were consistent with that of the naturally infected calves from whom the oocysts were originally derived.

3.6.1 Age-related clinical disease and oocyst shedding

The results of this study confirmed previous reports which demonstrated that livestock (calves and lambs) which were naïve to *Cryptosporidium* were still susceptible to the parasite at an older age (Harp et al., 1990; Ortega-Mora and Wright, 1994). In the present study the animals challenged at 6 weeks of age suffered from significantly less severe clinical disease than animals challenged at <1 week of age although they all still showed evidence of oocyst shedding. The

same is true of the lambs challenged in a previous study (Ortega-Mora and Wright, 1994). The lambs in that study were infected at 6 days and then re-challenged (with the same isolate and dose (1×10^6)) at 4 weeks of age. In parallel, a further two groups of lambs were infected once at 4 and 8 weeks of age respectively. Lambs which were naïve to *C. parvum* at 4 weeks still showed clinical disease (diarrhoea) and shed oocysts whereas lambs with prior exposure (infected at 1 week of age) had no clinical disease or (detectable) oocyst shedding when re-challenged at 4 weeks of age. This demonstrates that lambs can develop specific immunity to infection with this particular *C. parvum* isolate if they have previous exposure to the same parasite. When naïve lambs were challenged at 8 weeks of age none of the lambs showed evidence of clinical disease though some lambs did shed oocysts although this was over a shorter duration and lower numbers of oocysts were shed. This indicates that sometime between 4 (naïve lambs still susceptible to disease) and 8 weeks (naïve lambs not susceptible to disease) of age lambs develop an age-related resistance to *Cryptosporidium* infection.

In the present study we demonstrate that lambs become less susceptible to disease at around 6 weeks of age with a reduction in the percentage of animals showing clinical signs of disease to 17–33% from 50-100% depending on isolate despite an increase in challenge dose. This shows that as the animals increase in age they are becoming less susceptible to cryptosporidiosis with a reduction in disease severity at 6 weeks but no change in oocyst shedding. In a similar study in calves, Harp et al (1990) reported that when naïve calves were challenged with *C. parvum* at 1 or 3 months of age they remained fully susceptible to disease and all challenged animals showed clinical signs (diarrhoea) as well as oocyst shedding. Two of the animals which were initially challenged at 3 months were re-challenged at 6 months of age and neither showed any evidence of clinical disease or oocyst shedding. There were no calves which were naïve to *Cryptosporidium* challenged at this age so it is not known if the calves developed resistance to disease because of previous exposure or because of an age-related immune response. Based on this study it seems that, in calves, a previous challenge can protect against re-infection indicating that exposure of calves to *C. parvum* results in specific acquired immunity but the age at which they develop age-related immunity to disease is not known. However, as will be

reported later in this thesis, it is possible for calves with previous exposure to one isolate of *C. parvum* to become infected with, and shed oocysts of, another *C. parvum* isolate later in life (Chapter 5).

It is possible that this age-related resistance to symptomatic disease in lambs (6-8 weeks of age) and calves (>6 months) is related to changes within the animal gut. These changes usually occur at weaning or when the diet of the animal is changed. In the present study lambs were fed on UHT-treated milk 3 × per day for the first 2 weeks with *ad libitum* access to hay, pelleted lamb feed was made available from day 14 onwards. In the study by Ortega-Mora et al., (1994) lambs were fed on UHT-treated milk only for the first 20 days after which hay and lamb rations were made available. By day 45 of age the lambs had been weaned from milk completely. In the calf study (Harp et al., 1990) calves were fed a diet of milk replacer for 2.5 months with the addition of pelleted feed at 2 weeks of age and hay at 4 weeks of age. In addition, rumen fluid from five calves at 3.5 months of age was tested and showed some degree of function but not at the level of a fully mature adult bovine. The maturity of the rumen in cattle and sheep is affected by the age of weaning and diet (Quigley, III et al., 1985) . This ties in with the development of age-related resistance to disease in both previous studies (resistant to disease at 6 months (calves) and 8 weeks (lambs)) and the current study (not resistant at 6 weeks but mild clinical disease only). It is not known if an animal with mature gut function which has no prior exposure to *Cryptosporidium* would be susceptible to disease. From other studies, carried out as part of this PhD we know that calves can still remain susceptible to infection and can shed oocysts at 6 months of age (Chapter 5 and Chapter 6).

In mice age-related resistance to disease caused by *Cryptosporidium* has been well documented (Sherwood et al., 1982; Tarazona et al., 1997; Upton and Gillock, 1996) and it has been demonstrated that even when the mice have had no previous exposure to the parasite they were not susceptible to disease after 3 weeks of age. This age-related resistance in mice coincides with the maturation of the intestinal gut flora (about 3 weeks in mice) (Harp et al., 1992). It has also been noted that pigs become more susceptible to infection with *C. scrofarum* at around 5-8 weeks of age (Kvac et al., 2014), the authors suggested that this may

be due to changes in the gut at weaning (~6 weeks of age) which may make the gut easier for the parasite to invade.

3.6.2 Challenge dose, Clinical Disease and oocyst shedding

It appeared that when lambs received a higher infective dose (5×10^6) they began shedding oocysts sooner than when they received a lower infective dose (1×10^6). However, due to the age difference between the two dose groups it is not possible to attribute the shortened onset to oocyst shedding following infection at 6 weeks of age solely to the higher infective dose as age of the animal may also contribute to this effect. To clarify this a further study which focuses only on effect of age OR effect of infective dose on animals of the same age should be carried out. Lambs which received a secondary challenge at 6 weeks of age shed significantly more oocysts than lambs receiving a primary infection at this age. It is possible that this is because the lambs receiving a secondary challenge are still shedding oocysts from the primary infection they received at <1 week of age. It is also a possibility that the early onset of oocyst shedding which was seen in lambs receiving a higher dose may simply be due to some ingested oocysts passing through the animal.

Within each group in this study there were significant differences in individual lamb's responses to infection in terms of oocysts shed and clinical disease with the variation between some animals being considerable. Thirty-one percent of samples collected from lamb F (Group V) over the whole study were diarrhoeic compared with just 5.2% of samples from lamb C (Group V) and in Group III lamb F shed 1.01×10^{10} oocysts in total compared with lamb A (Group III) which shed 5.75×10^8 oocyts in total, a difference of 9.50×10^9.

Differences, sometimes considerable, in oocyst shedding (number of oocysts shed, duration of shedding) as well as clinical manifestation between individuals within groups have been noted in other *Cryptosporidium* infection trials in humans (Baxby et al., 1985; Chappell et al., 1996; Teunis et al., 2002), calves (Moore et al., 2003) and mice (Petry et al., 1995). This may be due to the different immunocompetence status of individual animals; all lambs in this study received some colostrum, either from their dams or artificial colostrum. The quality of each individual ewes colostrum was not known, nor was the quantity

that the individual lamb took. It is known that in other livestock and in humans the severity of disease depends very much on the immune status of the individual. Immunocompromised patients (human and animal) will suffer from more severe and prolonged disease than immunocompetent individuals (Baxby et al., 1985; Current et al., 1983; DuPont et al., 1995; Harp et al., 1992; Harp et al., 1988; Hojlyng and Jensen, 1988; Shepherd et al., 1988; Snyder et al., 1978).

We already know that the prepatent period is dependent on host species, *Cryptosporidium* species and infective dose (Tzipori et al., 1983). In this study we noted that following primary challenge at <1 week of age the animals in both Groups III & V did not begin shedding oocysts until 3-14 DPI (Group III (mean = 7.2, mode = 4, median = 5.5) Group V (mean = 9.8, mode = 14, median = 10)) whereas, when these animals were re-challenged at 6 weeks of age, with a higher dose, they began shedding oocysts much sooner, almost immediately following the oral challenge. It is not possible to determine exact number of DPI following secondary challenge as most animals did not completely cease shedding oocysts before being re-challenged. Although, it appears that 1-3 DPI the animals began shedding much higher numbers of oocysts than previously. The difference in the prepatent period following higher or lower doses has also been noted in other studies. Zambriski et al., (2013) carried out an experimental challenge in calves using doses of 25, 50, 100, 500, 1×10^3, 1×10^4, 1×10^5, and 1×10^6 *C. parvum* oocysts. They reported that calves receiving lower doses (25 and 50 oocysts) shed later than calves receiving higher doses (100 - 1×10^5 and 1×10^6) oocysts. Other studies report a mean prepatent period of 7.4 DPI in experimentally infected calves (challenged with 50, 100, 1000 and 10,000 *C. parvum* oocysts) with a longer prepatent period for animals receiving a lower dose and an increase in number of days of oocyst shedding in calves receiving a higher challenge dose and 16.3 DPI in naturally infected calves (Moore et al., 2003; O'Handley et al., 1999).

Not every sample produced from each animal was positive for oocysts indicating that oocyst production and excretion is not a constant process, similar results have been noted in experimental infections of human volunteers (Chappell et al., 1996). The observation in variation of oocysts shedding day to day could be as a result of the limitations of the detection method (62500 oocysts per gram of

faeces) used in this study. This suggests that for diagnostic purposes a single sample from a potentially infected individual, even if with clinical disease, may not be sufficient to detect oocysts and diagnose infection. To minimise the chances of missing potential positive samples total faecal output was collected and processed from all lambs in this study (during the clinical stage). In addition, the whole faecal sample was homogenised completely before a sub-sample was taken for analysis.

As we can see from the results of this study the amplification rate of the parasite in a young naïve animal is phenomenal; an animal infected with 1×10^6 oocysts is able to shed up to 2.73×10^9 potentially infectious oocysts in a two week period. In theory, this is enough to infect a further 2730 susceptible hosts with the same number of oocysts as the initial challenge. It is the amplification rate which leads to the rapid spread of disease within a herd or flock. From this we can see why, in a field setting, it is possible that older lambs, or even adults, may become subclinically infected with *Cryptosporidium* and maintain the parasite between lambing or calving periods.

3.6.3 *Cryptosporidium parvum* isolates

Despite the similarities in management on these farms the differences in disease between calves on the two farms is marked with calves on farm LH becoming very sick and calves on farm BB showing very little illness. If we take only the clinical manifestation of disease on the farms into consideration we may be able to conclude that the better early calf management (good quality colostrum within 2 h of birth, calf jackets) on farm BB means that the calves on this farm are better protected against disease in general and are better able to fight off infection. The results of the study described in this chapter indicate that these differences which were noted in naturally infected calves are mimicked the experimental infection trial in lambs. We found that isolate LH which caused more severe disease in naturally infected calves also was associated with an increased probability of diarrhoea and number of days of diarrhoea as well as a quicker onset to diarrhoea in the experimentally infected lambs and that isolate BB which seemed to cause an increase in oocyst output in naturally infected calves was shown to significantly affect the oocyst shedding of experimentally infected lambs.

Other studies have also reported variations in the severity of infection in humans and animals depending on the specific *Cryptosporidium* species and isolate (Cama et al., 2008; Cama et al., 2007). An experimental trial which involved infection of calves with four different *C. parvum* isolates (three derived from human patients and one calf derived) showed that there was a difference in disease severity between the isolates examined (Pozio et al., 1992). Although, with the added knowledge that we have now we cannot be sure that these isolates were a) truly *C. parvum* and not another species i.e. *C. hominis* (which is not infectious in calves (Morgan-Ryan et al., 2002)) and b) different isolates, in this study the oocysts were assumed to be different isolates of *C. parvum* based on morphology and clinical presentation in naturally infected hosts. Another study (Okhuysen et al., 1999) investigated the virulence of three *C. parvum* isolates in human volunteers (two calf derived and one horse derived) and found differences in ID_{50}, prepatent period and duration of oocyst shedding between the three isolates. These studies indicate that though host factors are very important and can be responsible for differences in individual response to infection with *C. parvum* it is also clear that there can be a difference in virulence and pathogenicity of particular isolates.

3.7 Conclusion

In summary, it appears that from the work carried out in this study that when lambs are initially challenged with *C. parvum* at <1 week of age the resulting disease is more severe than when lambs are challenged at 6 weeks of age. The fact that lambs which are naïve to *Cryptosporidium* still show mild clinical disease when challenged at 6 weeks of age with a higher challenge dose demonstrates that lambs do not develop complete immunity to disease with increasing age. The results of this study also show that infection with *C. parvum* does not provide complete protection against a homologous or heterologous challenge later in life. These results do not confirm a previous study which showed that lambs challenged at <1 week of age will be resistant to a homologous challenge at 1 month of age (Ortega-Mora and Wright, 1994) though in the present study any protection provided by prior challenge is masked by the effect of age. The lambs in this study were also challenged with a higher dose at 6 weeks of age whereas the lambs in the study by Ortega-Mora & Wright were given exactly the same challenge dose at 1 month of age. It is possible that had the lambs in this study received an identical challenge dose at 6 weeks of age to the one they received at <1 week of age the results may have been different. There may also be differences in the isolates used in these studies, the study by Ortega-Mora and Wright (1994) used the Moredun isolate (first isolated from a deer calf and then passaged in calves and lambs 4 × per year since 1987) and the present study used two different isolates (BB and LH) both isolated from calves and passaged once in neonatal lambs.

We have also demonstrated in this study that it is possible for different *C. parvum* isolates to behave differently in the host. From the results of this study we can report that one isolate (BB) resulted in fewer diarrhoeic samples in infected animals than another isolate (LH) which caused more severe and prolonged diarrhoea in infected animals. We also demonstrated that clinical disease does not directly correlate with oocyst output; animals challenged with isolate BB shed greater numbers of oocysts whilst showing milder clinical signs compared with animals infected with isolate LH. In some cases the oocyst shedding was completely asymptomatic and these asymptomatic animals could, in a field setting, be shedding several million potentially infective oocysts into

the environment where susceptible human and animal hosts could then become infected.

Further research in this area could be focused on determining the host factors which effect resistance to disease and why individuals experience different clinical manifestations of disease even when challenged with the same isolate and dose. In this study, there were differences in clinical disease and oocyst shedding between individuals from the same challenge groups. Some animals shed much larger numbers of oocysts than others in the same challenge group and some suffered from diarrhoea while others simply exhibited asymptomatic shedding. Differences in clinical symptoms have been reported in other studies (Jeckel and Williams, 2009; Lindsay et al., 1986; Santin, 2013; Vitovec et al., 2006; Zambriski et al., 2013a) but the reason for these differences is unknown, it is likely that it is due to differences in the immune response of the infected individuals. Understanding more about how and when animals mount an immune response to infection with *Cryptosporidium* will help in the development of suitable control measures.

Papers in preparation and presentations at conferences

1. Can infection with one genotype of *C. parvum* protect against infection with another – **5th International *Cryptosporidium* and *Giardia* conference** – Sweden, 2014
2. Can infection with one genotype of *C. parvum* protect against infection with another – **Exchange Visit** – Brazil, 2014

Chapter 4: Development of a molecular tool to differentiate between the most common species of *Cryptosporidium* in cattle

4.1 Introduction

For the work carried out for this thesis one of the first steps was to develop a quick, easy and reliable method for detection and speciation of *Cryptosporidium* species which may be found in cattle. This was to enable rapid identification of species present in faecal samples from calves and older cattle.

The most common methods for detection of *Cryptosporidium* in farm livestock are microscopy (in veterinary diagnostic laboratories) or by molecular detection (research laboratories); of the two methods molecular detection followed by sequencing or RFLP is much more sensitive and provides additional information (such as species or genotype) than microscopy which can only indicate the presence of the parasite (Chalmers and Katzer, 2013). One of the most common genes targeted for PCR detection of *Cryptosporidium* is the 18S rRNA gene which is located on a multi-copy DNA element meaning that the PCR is more sensitive than those which target a single copy gene for example actin, COWP or hsp70. The 18S rRNA gene is also one of the most commonly used genes for species discrimination and there are many 18S rRNA reference sequences available on the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). When using this gene it is necessary to carry out sequencing or PCR-RFLP to identify species in *Cryptosporidium* positive samples. These methods can be expensive, time consuming and it is very difficult to detect mixed infections as often only the most predominant species in a sample is detected (Cama et al., 2006).

A multiplex PCR test would enable the rapid speciation of a subset of *Cryptosporidium* species and previous attempts have been made to develop such a test (Lindergard et al., 2003; Patel et al., 1999; Santin and Zarlenga, 2009); the most recent of which can detect the four most commonly found *Cryptosporidium* species in cattle (Santin and Zarlenga, 2009). This test amplifies different sized fragments of the actin gene for the different species (*C. bovis* and *C. ryanae* both generate identically sized fragments of 300 bp and

further PCR tests and sequencing would be required to distinguish these species). This defeats the purpose of designing such a test and it would still be very difficult if not impossible to detect mixed infections if both of these species were present.

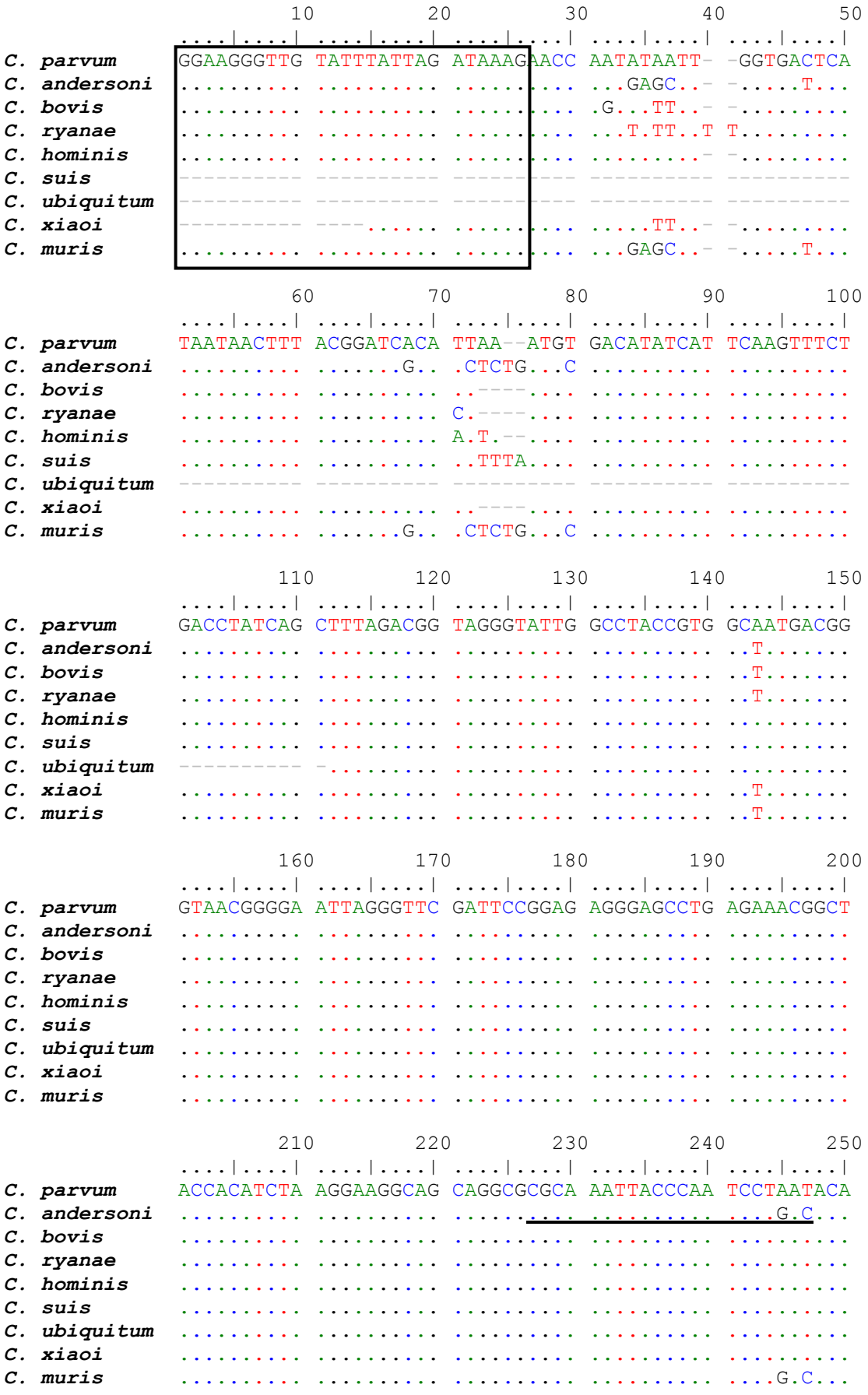
4.2 Aims

1) Develop a multiplex PCR that enables the identification of the four most common *Cryptosporidium* species found in cattle in single and mixed infections as well as identify any non-cattle adapted species

4.3 Materials and Methods

4.3.1 Design of *Cryptosporidium* species specific primers

Alignments of multiple (between 50 and 100 for each species) 18S rRNA gene sequences for *Cryptosporidium* species (*C. parvum*, *C. bovis*, *C. ryanae*, *C. andersoni*, *C. ubiquitum*, *C. hominis*, *C. muris*, *C. xiaoi* and *C. suis*) were made using the BioEdit Software Program (www.mbio.ncsu.edu/bioedit/bioedit.html) to identify representative sequences for each species. ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2) alignments of the representative sequences were made and regions for species specific primers were identified which would produce amplicons of different sizes for each species when used with the 18S rRNA reverse primer (AL3032) (Xiao et al., 2000a). The location of the primers used are shown in Figure 4-1; the first set of primers (AL1598 and AL3032) are those described by Xiao et al., 2000a and amplify a 840 bp genus specific fragment, the second primer set (CaF and AL3032) amplify a 625 bp fragment from *C. andersoni*; the third set (CrF and AL3032) amplify a 415 bp fragment from *C. ryanae*; the forward primer in this set contains a degenerate base (R) where the *C. ryanae* sequence contains either an 'A' or a 'G' ; the fourth set (CphF and AL3032) amplify a 305 bp fragment from *C. parvum, C. hominis* and *C. suis* which can be later distinguished with an additional PCR if desired and the final set of primers (CbF and AL3032) amplify a 241 bp fragment from *C. bovis*. The primer sequences are shown in Table 4-1.


10 20 30 40 50
C. parvum GGAAGGGTTG TATTTATTAG ATAAAGAACC AATATAATT- -GGTGACTCA
C. andersoni .....GAGC..- -.....T...
C. bovis .....G...TT..- -.....
C. ryanae .....T.TT..T T.....
C. hominis .....- -.....
C. suis
C. ubiquitum
C. xiaoi .....TT..- -.....
C. muris .....GAGC..- -.....T...
60 70 80 90 100
C. parvum TAATAACTTT ACGGATCACA TTAA--ATGT GACATATCAT TCAAGTTTCT
C. andersoni .....G.. .CTCTG...C .....
C. bovis .....
C. ryanae .....C.----.... .....
C. hominis .....A.T.--.... .....
C. suis .....TTTA.... .....
C. ubiquitum
C. xiaoi .....
C. muris .....G.. .CTCTG...C .....
110 120 130 140 150
C. parvum GACCTATCAG CTTTAGACGG TAGGGTATTG GCCTACCGTG GCAATGACGG
C. andersoni .....T.....
C. bovis .....T.....
C. ryanae .....T.....
C. hominis .....
C. suis .....
C. ubiquitum -.....
C. xiaoi .....T.....
C. muris .....T.....
160 170 180 190 200
C. parvum GTAACGGGGA ATTAGGGTTC GATTCCGGAG AGGGAGCCTG AGAAACGGCT
C. andersoni .....
C. bovis .....
C. ryanae .....
C. hominis .....
C. suis .....
C. ubiquitum .....
C. xiaoi .....
C. muris .....
210 220 230 240 250
C. parvum ACCACATCTA AGGAAGGCAG CAGGCGCGCA AATTACCCAA TCCTAATACA
C. andersoni .....G.C...
C. bovis .....
C. ryanae .....
C. hominis .....
C. suis .....
C. ubiquitum .....
C. xiaoi .....
C. muris .....G.C...

```
                        260        270        280        290        300
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          GGGAGGTAGT GACAAGAAAT AACAATACAG GACTT-TT-T GGTTTTGTAA
C. andersoni       .......... .......... .......... .G.C.-AA-C ...C......
C. bovis           .......... .......... .......... A..C.-.A-C ..........
C. ryanae          .......... .......... .......... AG.C.-.A-C ..........
C. hominis         .......... .......... .......... .....-..-. ..........
C. suis            .......... .......... .......... .....T..-. A.........
C. ubiquitum       .......... .......... .......... .....TAAA. A.........
C. xiaoi           .......... .......... .......... A..C.-.A-C ..........
C. muris           .......... .......... .......... .G.C.-AA-C ...C......

                        310        320        330        340        350
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          TTGGAATGAG TTAAGTATAA ACCCCTTTAC AAGTATCAAT TGGAGGGCAA
C. andersoni       .......... .G........ .......... G......... ..........
C. bovis           .......... .......... .......A.. .......... ..........
C. ryanae          .......... .......... .......A.. .......... ..........
C. hominis         .......... .......... .......... .......... ..........
C. suis            .......... .......... .........C. .......... ..........
C. ubiquitum       .......... .......... .......... .......... ..........
C. xiaoi           .......... .......... .......A.. .......... ..........
C. muris           .......... .G........ .......... G......... ..........

                        360        370        380        390        400
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          GTCTGGTGCC AGCAGCCGCG GTAATTCCAG CTCCAATAGC GTATATTAAA
C. andersoni       .......... .......... .......... .......... ..........
C. bovis           .......... .......... .......... .......... ..........
C. ryanae          .......... .......... .......... .......... ..........
C. hominis         .......... .......... .......... .......... ..........
C. suis            .......... .......... .......... .......... ..........
C. ubiquitum       .......... .......... .......... .......... ..........
C. xiaoi           .......... .......... .......... .......... ..........
C. muris           .......... .......... .......... .......... ..........

                        410        420        430        440        450
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          GTTGTTGCAG TTAAAAAGCT CGTAGTTGGA TTTCTGTTAA TAATTTATAT
C. andersoni       .......... .......... .......... ........GT AT.A..T...
C. bovis           .......... .......... .......AAT C........- --....T...
C. ryanae          .......... .......... .......AAT .........- --....T...
C. hominis         .......... .......... .......... .......... ..........
C. suis            .......... .......... T......... .......... ..........
C. ubiquitum       .......... .......... .......... .......... ..........
C. xiaoi           .......... .......... .......AAT C........- --....T...
C. muris           .......... .......... .......... ........GT AT.A.CT-..

                        460        470        480        490        500
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          AAAATATTTT GA-TGAATAT TTAT-ATAAT ATTAACATAA TTCATATTAC
C. andersoni       .--.....AC C--AAGG..A ....T...T. ..C.....CC ...C.....T
C. bovis           .T-...A.A. C--ACG.... ....-..... .......... ..........
C. ryanae          .T-.C.A.GC T--ACGG... ....-..... .......... ..........
C. hominis         .......... ..-....... ....-..... .......... ..........
C. suis            .T........ T----..... ....-..... .......... ..........
C. ubiquitum       .T........ ATT--..... ....-...G. .......... ..........
C. xiaoi           .T-.C.A.A. C--ACG.... ....-..... .......... ..........
C. muris           .--.....AC T--AAGG... A...T...T. ..C.....CC ...C.....T
```

```
                          510         520         530         540         550
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          TATATATTTT ---AGTATAT GAAATTTTAC TTTGAGAAAA TTAGAGTGCT
C. andersoni       AT.-CTAAA. ----A....G .......... .......... ..........
C. bovis           .T.T..G--- -----..... ....C..... .......... ..........
C. ryanae          .T.T..G--- -----..... ....C..... .......... ..........
C. hominis         ...T.T.... TTT....... .......... .......... ..........
C. suis            ....AT.... ATT....... .......... .......... ..........
C. ubiquitum       ....--.... AT-....... .......... .......... ..........
C. xiaoi           .T.T..G--- -----..... ....C..... .......... ..........
C. muris           AT.TCTAAA. ----A....G ....C..... .......... ..........

                          560         570         580         590         600
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          TAAAGCAGGC ATATGCCTTG AATACTCCAG CATGGAATAA TAT-TAAAGA
C. andersoni       .......... .AC....... .......... .......... ..AG...G..
C. bovis           .......... TAT....... .......... .......... ...-...G..
C. ryanae          .......... TAT....... .......... .......... ...-...G..
C. hominis         .......... .......... .......... .......... ...-......
C. suis            .......... .......... .......... .......... ...-A.....
C. ubiquitum       .......... ..TA...... .......... .......... ...-A.....
C. xiaoi           .......... TAT....... .......... .......... ...-...G..
C. muris           .......... .AC....... .......... .......... ..AG...G..

                          610         620         630         640         650
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          TTTTTATCTT TCTTATTGGT TCTAAGATAA GAATAATGAT TAATAGGGAC
C. andersoni       C....G.... .......... ....G..C.. A.G.....G. ..........
C. bovis           .......TC. .......... ....GA.... A...G..... ..........
C. ryanae          .......TC. .......... ....GA.... A......... ..........
C. hominis         .......... .T........ .......... .......... ..........
C. suis            .......... .T........ .......... A......... ..........
C. ubiquitum       .......... .T........ .......... A......... ..........
C. xiaoi           .......TC. .......... ....GA.... A...G..... .......A..
C. muris           C....G.... .......... ....G..C.. A.G.....G. ..........

                          660         670         680         690         700
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          AGTTGGGGGC ATTTGTATTT AACAGTCAGA GGTGAAATTC TTAGATTTGT
C. andersoni       .......... ...C...... .....C.... .......... ..........
C. bovis           .......... .......... .......... .......... ..........
C. ryanae          .......... .......... .......... .......... ..........
C. hominis         .......... .......... .......... .......... ..........
C. suis            .......... .......... .......... .......... ..........
C. ubiquitum       .......... .......... .......... .......... ..........
C. xiaoi           .......... .......... .......... .......... ..........
C. muris           .......... ...C...... .....C.... .......... ..........

                          710         720         730         740         750
                   ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum          TAAAGACAAA CTAATGCGAA AGCATTTGCC AAGGATGTTT TCATTAATCA
C. andersoni       .......G.. ...C...... .......... .......... ..........
C. bovis           .......... ...C...... .......... .......... ..........
C. ryanae          .......... ...C...... .......... .......... ..........
C. hominis         .......... .......... .......... .......... ..........
C. suis            .......... ...G...... .......... .......... ..........
C. ubiquitum       .......... ...G...... .......... .......... ..........
C. xiaoi           .......... ...C...... .......... .......... ..........
C. muris           .......G.. ...C...... .......... .......... ..........
```

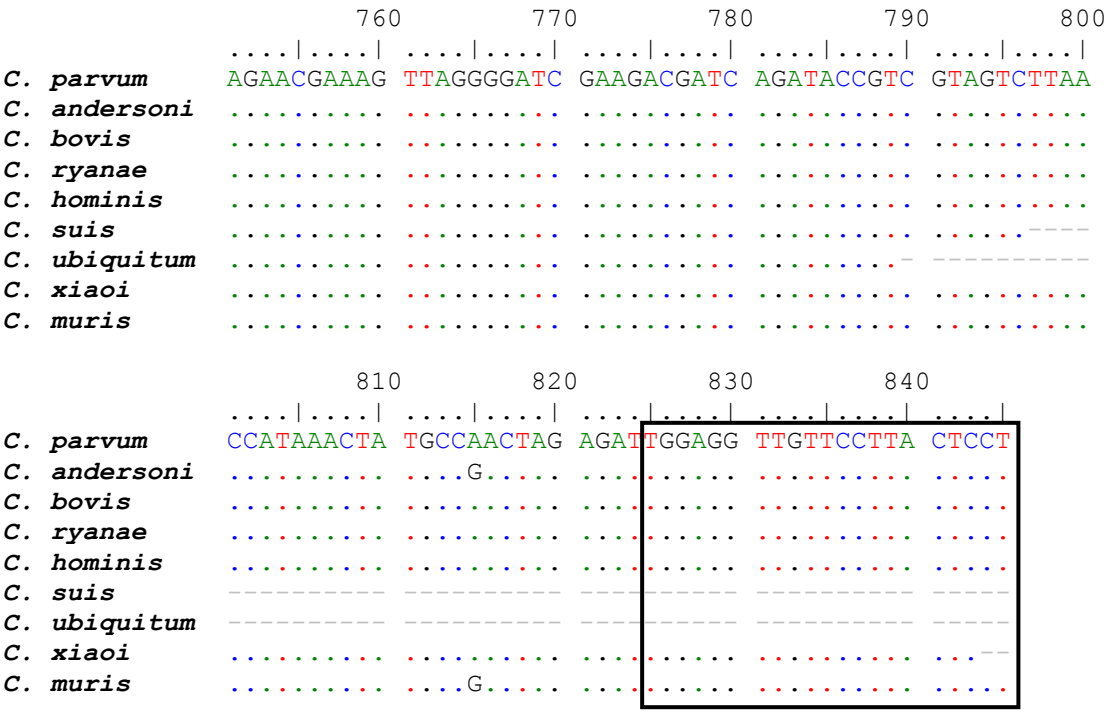


Figure 4-1: Positions of species specific primers on the 18S rRNA gene plus the location of the 18S rRNA internal primers.
The primers designed amplify a 625bp fragment for *C. andersoni*, 415bp fragment for *C. ryanae*, 305bp fragment for *C. parvum*, *C. hominis* and *C. suis* and a 241bp fragment for *C. bovis*. Accession numbers for each species were: *C. andersoni* (FJ463187), *C. ryanae* (JN400880), *C. parvum* (AF164102), *C. bovis* (HQ179572), *C. hominis* (DQ286403), *C. suis* (AB694726), *C. xiaoi* (GU014553.1), *C. muris* (AF381167.1) and *C. ubiquitum* (KC608029.1).

Table 4-1: Primer sequences and size of amplicon produced with each primer pair

Primer	Sequence 5'-3'	Fragment Size (bp)	Species Detected
AL1687 (EF)	TTCTAGAGCTAATACATGCG	1370	Genus
AL1691 (ER)	CCCATTTCTTCGAAACAGGA		Specific
AL1598 (IF)	GAAGGGTTGTATTTATTAGATAAAG	840	Genus
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA		Specific
CaF	GCAAATTACCCAATCCTGAC	625	*C. andersoni*
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA		
CrF	TGTTAATTTTTATATACAAT**R***CTACGG	415	*C. ryanae*
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA		
CphF	AGAGTGCTTAAAGCAGGCATA	305	*C. parvum*
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA		
CbF	CTTCTTATTCCTTCTAGAATAAAAATG	241	*C. bovis*
AL3032 (IR)	AAGGAGTAAGGAACAACCTCCA		

*The degenerate base in the *C. ryanae* primer is shown in bold and underlined

4.3.2 Faecal Sample Collection and DNA extraction

Faecal samples (n=498) were collected from 308 adult cattle and 190 calves from 21 beef farms in Aberdeenshire and Caithness, Northeast Scotland in Spring 2011 as part of another study. The faecal samples were collected by the farmers and sent to the Moredun Research Institute by their vet practices.

DNA was extracted from the calf faeces processing with a QIAamp DNA Stool Minikit (Qiagen) according to the manufacturer's instructions except that 10 × freeze-thaw cycles in liquid nitrogen were carried out after adding 200 µg sample to lysis buffer (provided in the kit).

Adult faeces were subjected to a sedimentation step prior to DNA extraction. Briefly, 3 g of faeces was added to a 50 ml tube (A) and resuspended in 25 ml dH_2O before vortexing vigorously. The solution was left to settle for 5 mins and the supernatant removed and added to a second tube (B). The pellet in tube A was resuspended with 25 ml dH_2O and the settlement step repeated, the supernatant was removed from tube A and added to tube B. The tube containing the supernatant was centrifuged at 1000 ***x g*** for 5 mins and the supernatant discarded, 200 µg of the resultant pellet was added to lysis buffer and subjected

to 10 × freeze-thaw cycles in liquid nitrogen followed by processing by QIAamp DNA Stool Minikit (Qiagen) according to the manufacturer's instructions.

All 498 samples were tested for the presence of *Cryptosporidium* parasite DNA using the 18S genus specific PCR, 4.13% (n=13) of the samples from adult cattle and 60.5% (n=115) of the samples from calves tested positive for *Cryptosporidium*. These samples were used to test the nested species specific multiplex – Polymerase Chain Reaction (nssm-PCR).

4.3.2.1 Testing of individual species specific primers

First round reactions were carried out as described in section 2.6.1 using DNA from known positive samples for each of the four species. Secondary reactions were carried out to test each individual species specific forward primer with the reverse 18S rRNA primer (AL3032). First round PCR products were diluted with 50 µl dH_2O and 1 µl of the dilution used as a template in a reaction containing 2.5 µl 10× PCR buffer (45 mM Tris-HCL pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 4.4 µM EDTA, 113 µg ml-1 BSA, 1 mM of each of the four deoxyribonucleotide triphosphates) (MacLeod et al., 1999), 0.5 units BioTaq (Bioline, UK) and 10 µM forward and reverse primers. The final volume of each reaction was made up to 25 µl using dH_2O.

Cycling conditions were 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s on a temperature gradient of 49 - 59°C and 1 min at 72°C. The final extension was 7 min at 72°C. Second round PCR products were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2200.

4.3.2.2 Field Samples

Primers were tested on a panel of DNA samples which included: known positive samples for *C. parvum, C. andersoni, C. bovis* and *C. ryanae* from naturally infected cattle (n=128). Results from the nssm-PCR were compared with sequence data for some of these samples.

4.3.2.3 Optimisation of PCR cycling conditions

The first round of the nssm-PCR was carried out in a 25 µl reaction as described in section 4.3.2.1 using 10 µM of each primer and 3 µl of template DNA in single and multiple species combinations. The first round products were diluted with 50 µl dH_2O and 1 µl of the dilution used as template in the 2nd round. Second round reactions were carried out in a 25 µl reaction as described in section 4.3.2.1. Primers were tested on a temperature gradient to determine the optimum annealing temperature. Cycling conditions were 3 minutes at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s on a temperature gradient of 49 - 59°C and 1 minute at 72°C. The final extension was 7 minutes at 72°C. Second round PCR products were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2200.

4.3.3 Sequence Analysis

To confirm the results of the nssm-PCR when testing field samples, sequence analysis of the 18S rRNA amplicon was carried out. Sequencing of PCR products was carried out by GATC (GATC, Germany) using their "Light-run" service. Five microlitres of purified PCR product was sent at a concentration of between 20-80 ng/µl with 5 µl of the appropriate primer at a concentration of 5 pmol/µl. In some circumstances, instead of the light-run service, unpurified PCR products were sent to GATC for sequencing using their "Supremerun96" sequencing option which includes PCR purification. With this service 20 µl PCR product was sent with a separate 1.5 ml tube containing 50 µl of the appropriate primer at a concentration of 10 pmol/µl. Sequences were analysed using BioEdit Sequence Alignment Software (www.mbio.ncsu.edu/bioedit/bioedit.html) and Chromas Lite (www.technelysium.com).

4.4 Results

4.4.1 Primer Design

The primer designed for *C. parvum* will also amplify *C. hominis* and *C. suis*, the *C. andersoni* primer will also amplify *C. muris* and the *C. bovis* primer will amplify *C. xiaoi* as the 18S region of these species are very similar. *Cryptosporidium suis* and *C. hominis* have only been found very rarely in cattle (Fayer et al., 2006; Smith et al., 2005) and *C. muris* and *C. xiaoi* have not yet been identified in cattle.

4.4.2 Individual Species PCR

Amplicons of the expected size for each species were obtained when tested in a single species PCR with the species specific forward primer and the 18S rRNA reverse primer.

4.4.3 Optimisation of PCR cycling conditions

An annealing temperature of 56°C was selected as the optimum temperature for all primers in the mix.

4.4.4 Nested Species Specific Multiplex PCR

Amplicons of the expected size for each of the four species (*C. andersoni, C. ryanae, C. parvum* and *C. bovis* – individually and mixed combinations) were obtained using the nssm-PCR assay (Figure 4-2) from a variety of cattle samples.

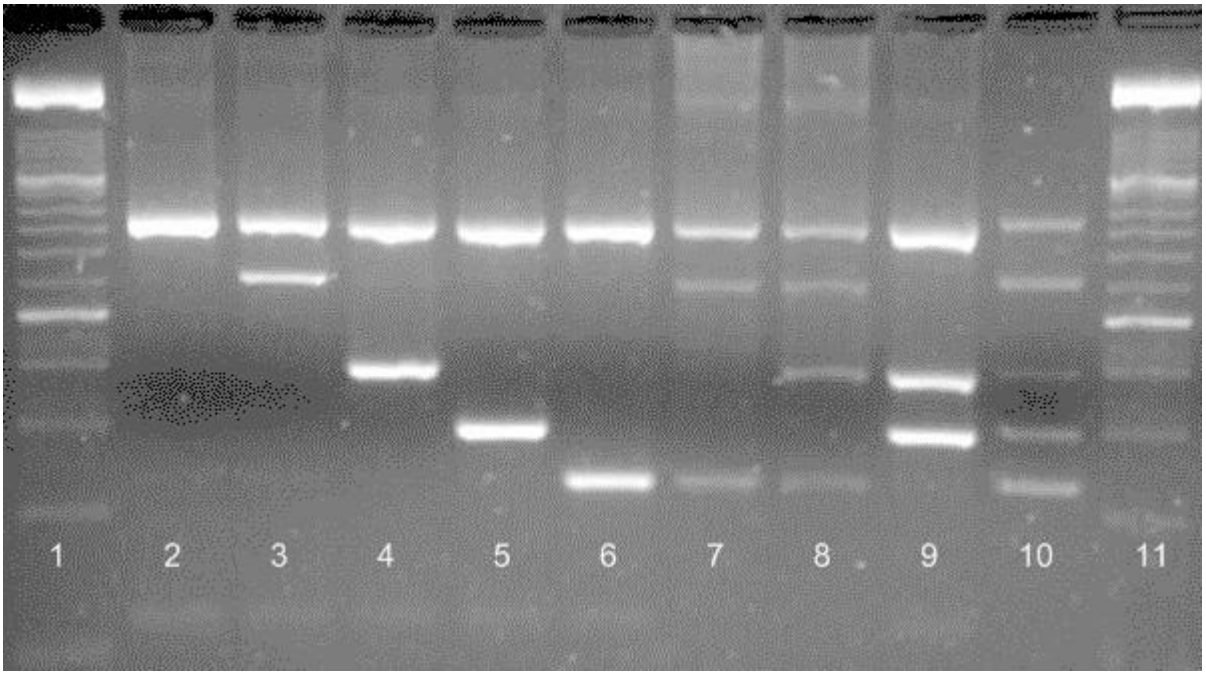


Figure 4-2: Detection of species specific amplicons on a 2% agarose gel stained with GelRedTM (Biotium, UK).
Lane 1, 100bp marker XIV (Roche); lane 2, 18S rRNA genus specific amplicon (840 bp); lane 3, *C. andersoni* (625 bp); lane 4, *C. ryanae* (415 bp); lane 5, *C. parvum* (305 bp); lane 6, *C. bovis* (241 bp); lane 7, *C. andersoni* and *C. bovis*; lane 8, *C. andersoni*, *C. ryanae* and *C. bovis*; lane 9, *C. ryanae* and *C. parvum*; lane 10, nssm-PCR molecular marker; lane 11, 100bp marker XIV (Roche). Amplicons in lanes 2-6 were produced using cloned DNA and lanes 7-9 were from naturally infected cattle.

From the 128 samples from naturally infected cattle (1 day – adult) from farms in Aberdeenshire and Caithness the expected amplicons for all four cattle species (*C. parvum* (n=92), *C. andersoni* (n=1), *C. ryanae* (only in mixed infections) and *C. bovis* (n=13)) were observed as were various combinations of mixed infections (Figure 4-2). Occasionally an additional non-specific band appeared with the amplicon for *C. parvum* but of a different size to the species specific bands. The results of the nssm-PCR were compared with sequence data for the 18S rRNA gene of these samples. In all cases the single species of *Cryptosporidium* identified by nssm-PCR matched the species identified by sequencing. In addition, the nssm-PCR identified mixed infections in 14 of the samples examined. The mixed infections included; *C. bovis, C. parvum* and *C. ryanae* (n=1), *C. ryanae* and *C. bovis* (n=5), *C. parvum* and *C. bovis* (n=1), *C. parvum* and *C. ryanae* (n=1) and *C. andersoni and C. ryanae* (n=6). These mixed infections were not identified by sequencing. Eight of the samples failed to amplify using the nssm-PCR and three produced unreadable trace files when sequenced (Table 4-2).

Table 4-2: Comparison of results of nssm-PCR with sequencing of 18S rRNA gene.

Sample Type	18S Sequence Result	18S Multiplex Result
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*failed sequencing*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*did not amplify*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*did not amplify*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*

Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis, C. parvum + C. ryanae*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. bovis*	*C. ryanae + C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. ryanae + C. bovis*
Calf (7-14 days)	*C. parvum*	*C. ryanae + C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. bovis*	*C. bovis + C. ryanae*
Calf (7-14 days)	*C. bovis*	*C. bovis + C. parvum*

Calf (7-14 days)	*C. suis*-like	*C. parvum + C. ryanae*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*failed sequencing*	*did not amplify*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. bovis*	*C. bovis + C. ryanae*
Calf (7-14 days)	*C. bovis*	*C. bovis*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*failed sequencing*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*

Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Calf (7-14 days)	*C. parvum*	*C. parvum*
Adult	*C. parvum*	*C. parvum*
Adult	*C. andersoni*	*C. andersoni*
Adult	*C. parvum*	*did not amplify*
Adult	*C. parvum*	*did not amplify*
Adult	*C. suis*-like	*did not amplify*
Adult	*C. andersoni*	*did not amplify*
Adult	*C. andersoni*	*C. andersoni + C. ryanae*
Adult	*C. andersoni*	*C. andersoni + C. ryanae*
Adult	*C. andersoni*	*C. andersoni + C. ryanae*
Adult	*C. andersoni*	*C. andersoni + C. ryanae*
Adult	*C. andersoni*	*C. andersoni + C. ryanae*
Adult	*C. andersoni*	*did not amplify*
Adult	*C. andersoni*	*C. andersoni + C. ryanae*

4.5 Discussion

In this chapter the development of a multiplex PCR test which allows the discrimination of four of the most commonly detected *Cryptosporidium* species of cattle (some of which are or may pose a risk to human health) is described. The test allows the differentiation of these species by generating distinct amplicons of the 18S rRNA gene: *C. andersoni* (625 bp), *C. ryanae* (415 bp), *C. parvum* (305 bp) and *C. bovis* (241 bp) in addition to a *Cryptosporidium* genus specific amplicon (840 bp). Currently the most popular method of species identification in *Cryptosporidium* positive samples is gene sequencing of the 18S rRNA gene. This method is very sensitive and can detect a large range of *Cryptosporidium* species and genotypes but is limited when mixed infections occur as it has been shown that in a mixed sample either the dominant *Cryptosporidium* species is preferentially amplified by PCR meaning that only one species will be identifiable by sequencing or the sequence is unreadable (Cama et al., 2006; Santin et al., 2004; Silverlas et al., 2010). The multiplex PCR described here allows the detection of mixed infections of up to four different common cattle species of *Cryptosporidium* and is an excellent method for detecting underlying infections of zoonotic *C. parvum* in calves which may have higher burdens of the less-pathogenic *Cryptosporidium* species.

In addition to the easy and quick visual differentiation of the four common cattle species other species of *Cryptosporidium* may be identified by sequencing of the 18S rRNA gene when a fragment responding to the *Cryptosporidium* genus specific amplicon (840 bp) is amplified with no species specific amplicons. At present, using this method, it is not possible to distinguish between *C. parvum*, *C. hominis* and *C. suis* however, as *C. hominis* and *C. suis* have rarely been detected in cattle (Park et al., 2006; Smith et al., 2005) there is, at this stage, no real need to distinguish between these two species. If necessary these species could be differentiated using a further PCR or by sequence analysis.

The advantages of the PCR protocol described here over other methods used for species discrimination (PCR-RFLP, sequencing) are: (1) the ability to detect mixed infections which may be missed by other methods; (2) the ability to identify other *Cryptosporidium* species by further sequencing when none of the cattle adapted species are detected; (3) the ability to test large numbers of

samples quickly and easily, and; (4) the ability to detect subclinical infections of the zoonotic species *C. parvum* in animals shedding high numbers of non-zoonotic species.

As well as being used extensively throughout this PhD thesis this molecular tool has also been used to identify the species of *Cryptosporidium* present in faeces from adult cattle and their calves as part of a whole catchment study in the North East of Scotland (Wells et al., 2015).

Papers in preparation and presentations at conferences

1. **Thomson, S.,** Innes, E.A., Jonsson, N., Katzer, F., Development of a Nested Species Specific Multiplex PCR for the detection of bovine adapted *Cryptosporidium* species. (Submitted)

Chapter 5: *Cryptosporidium* species and subtypes found in calves from birth to one year

5.1 Introduction

Cryptosporidium parvum is one of the most important causes of neonatal enteritis in calves not only in the UK but worldwide (Mosier and Oberst, 2000). It is known that cattle are commonly infected with four distinct species of *Cryptosporidium* and that infection with these species tends to follow an age-related distribution with *C. parvum* being predominantly found in pre-weaned calves, *C. bovis* and *C. ryanae* in older calves (<2 months) and *C. andersoni* in adult cattle (Fayer et al., 2006; Santin et al., 2004). There are some exceptions to this with *C. bovis* and *C. ryanae* being found in pre-weaned calves in some countries (France and Sweden) (Rieux et al., 2013; Silverlas et al., 2010) although these species have not been associated with clinical disease (Fayer et al., 2008; Fayer et al., 2005).

Recent advances in typing and subtyping methodologies has allowed more detailed studies into the dynamics of *C. parvum* infection in cattle and humans. The most common subtyping method is sequencing of the 60-kDa glycoprotein (*gp60*) gene, so far, at least 14 subtype families for *C. parvum* have been identified (IIa-IIo (there is no IIj)) (Wang et al., 2014a). Some of the subtype families appear to be exclusively anthroponotic (IIc and IIe) (Cama et al., 2008; Hijjawi et al., 2010; Sulaiman et al., 2005) and others are major zoonotic subtypes (IIa and IId) (Alves et al., 2001; Alves et al., 2003). The remaining subtype families have been found less commonly in animals and humans throughout the world. It has been demonstrated previously that most farms have one predominant *gp60* subtype within their calves which persists over time (Brook et al., 2009).

At present there is limited knowledge of the species and subtypes of *Cryptosporidium* found in cattle on Scottish farms. Particularly little is known about the shedding profiles (age and duration) of the different species found in cattle. Until more is understood about the species and subtypes which calves shed at different times throughout their lives it is difficult to develop effective strategies to control the parasite. Knowing when an animal is likely to pick up

the infection, and when they will be infective to others is hugely important as this will allow targeted control measures to help reduce the impact of cryptosporidiosis in young animals. This part of the thesis aims to address some of these questions by carrying out a detailed longitudinal study in calves.

5.2 Aims

1) Identify the species of *Cryptosporidium* that are shed by calves over the initial six weeks of life and then again at set points over one year

2) Determine the lengths of the intervals over which each of these species are shed

3) Determine how the prevalence of each species changes over time

4) Identify any periods during which the numbers of shed oocysts are significantly higher than average for the entire shedding period

5.3 Materials and Methods

5.3.1 Sample Collection

Faecal samples were collected from 25 calves on a dairy farm in Midlothian from the day of birth until six weeks of age, three times per week (Monday, Wednesday and Friday) as described in section 2.1.1. The calves were kept under the normal working conditions of this farm with regards to housing (kept in groups of ~20 in straw bedded pens), feeding (fed milk replacer with *ad libitum* access to pelleted calf feed, hay and water) and veterinary treatment. During this time none of the calves were treated specifically for scour or *Cryptosporidium*. The calves were kept with their mothers for one or two days then moved to a small group (5-8 calves) for one week then moved to a larger indoor straw bedded pen with approximately 20 calves in each pen for the remaining five weeks. After this time the calves were moved, and groups mixed, to a different, indoor straw bedded pen. Samples were collected from the same calves again at three, six, nine and twelve months of age where possible. The total number of samples collected was 426. The samples were mixed thoroughly and aliquoted into bijoux tubes and stored at -20°C for further processing.

5.3.2 Sample processing and DNA extraction

Faecal samples from calves up to, and including, 9 months were processed as described in section 2.5.2 and samples from calves aged 12 months as described in sections 2.4.1 and 2.5.1 to obtain DNA for PCR amplification.

5.3.3 PCR amplification of the 18S rRNA gene

All DNA samples were amplified using the 18S PCR described in section 2.6.1 and visualised on a 1.5% agarose gel stained with GelRed (Biotium, UK). All reactions were carried out in triplicate and a sample was considered positive if a band corresponding with ~840 bp was seen in 1/3 triplicates. Several controls, both negative (no template and extraction controls) and positive (*C. andersoni, C. parvum, C. bovis* and *C. ryanae* DNA) were run with each set of reactions. A positive result was only accepted when all negative controls were negative and positive controls were positive.

5.3.4 Species identification by nssm-PCR (nested species specific multiplex-PCR)

For the differentiation of *Cryptosporidium* species an nssm-PCR was carried out using the first round PCR product from the 18S PCR (see section 2.6.1) as a template. The PCR products were visualised on a 1.5% agarose gel stained with GelRed (Biotium, UK). All reactions were carried out in triplicate and a sample was considered positive if a band corresponding with 840 bp and any combination of 625 bp, 415 bp, 305 bp or 241 bp was seen in at least 1/3 lanes. Positive controls (obtained from cloned DNA from oocysts collected from naturally infected cattle as part of another study) for each *Cryptosporidium* species tested for were included as well as extraction controls and no template controls. A positive result was only accepted when all negative controls were negative and all positive controls were positive.

5.3.5 PCR amplification and sequencing of the *gp60* gene for *C. parvum* subtyping

Subtyping of *C. parvum* positive samples was carried out by amplification and sequencing of the *gp60* gene (see sections 2.6.3 and 2.7). The PCR products were visualised on a 1.5% agarose stained with GelRed (Biotium, UK). Reactions were originally carried out in duplicate and if amplification was not successful the reactions were repeated. A sample was considered positive if a band corresponding with ~375 bp was seen. Positive, negative and extraction controls were included for each set of reactions. A positive result was only accepted when all negative controls were negative and all positive controls were positive.

gp60 positive samples were sequenced in the forward and reverse orientation by GATC Biotech (GATC, Germany) as described for “supremerun96” sequencing in section 2.7.3. The sequences were analysed using Chromas Lite (version 2.01 Technelysium) and the subtypes named as according to (Sulaiman et al., 2005).

5.4 Results

5.4.1 Prevalence of *Cryptosporidium* in calves from birth to twelve months

In total 426 faecal samples were collected from calves on the study farm (Table 5-1).

Table 5-1: Number of samples collected at each time point

Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	3 Months	6 Months	9 Months	12 Months
64	62	63	56	57	57	17	16	17	17

All 426 samples were tested for the presence of *Cryptosporidium* parasite DNA using the 18S genus specific PCR and the overall percentage of positive samples for the whole study was 59.7% (n=255). In the first six weeks of life each calf tested positive for *Cryptosporidium* on at least six occasions, giving a period prevalence of 100% and the overall percentage of samples positive in calves <6 weeks was 64% (n=232). The earliest observation of oocyst shedding occurred at 2 d, which was observed in three calves. In several of the calves the first observation of oocyst shedding occurred at day 4 (4 animals) or day 6 (4 animals). Peak shedding of *Cryptosporidium* oocysts occurred in week 3 with 100% of the calves tested shedding oocysts. Oocyst shedding tailed off towards the end of week 6. Samples from the calves at 3, 6, 9 and 12 months showed a prevalence of 24%, 63%, 41% and 18.65% respectively (Figure 5-1). Five of the animals did not test positive for *Cryptosporidium* at any point after the initial 6 week period and no animals tested positive at all age groups though two tested positive at <6 weeks, 3, 6 and 9 months.

The percentage of positive samples collected from each calf ranged from 38.9% to 87.5% (Figure 5-2).

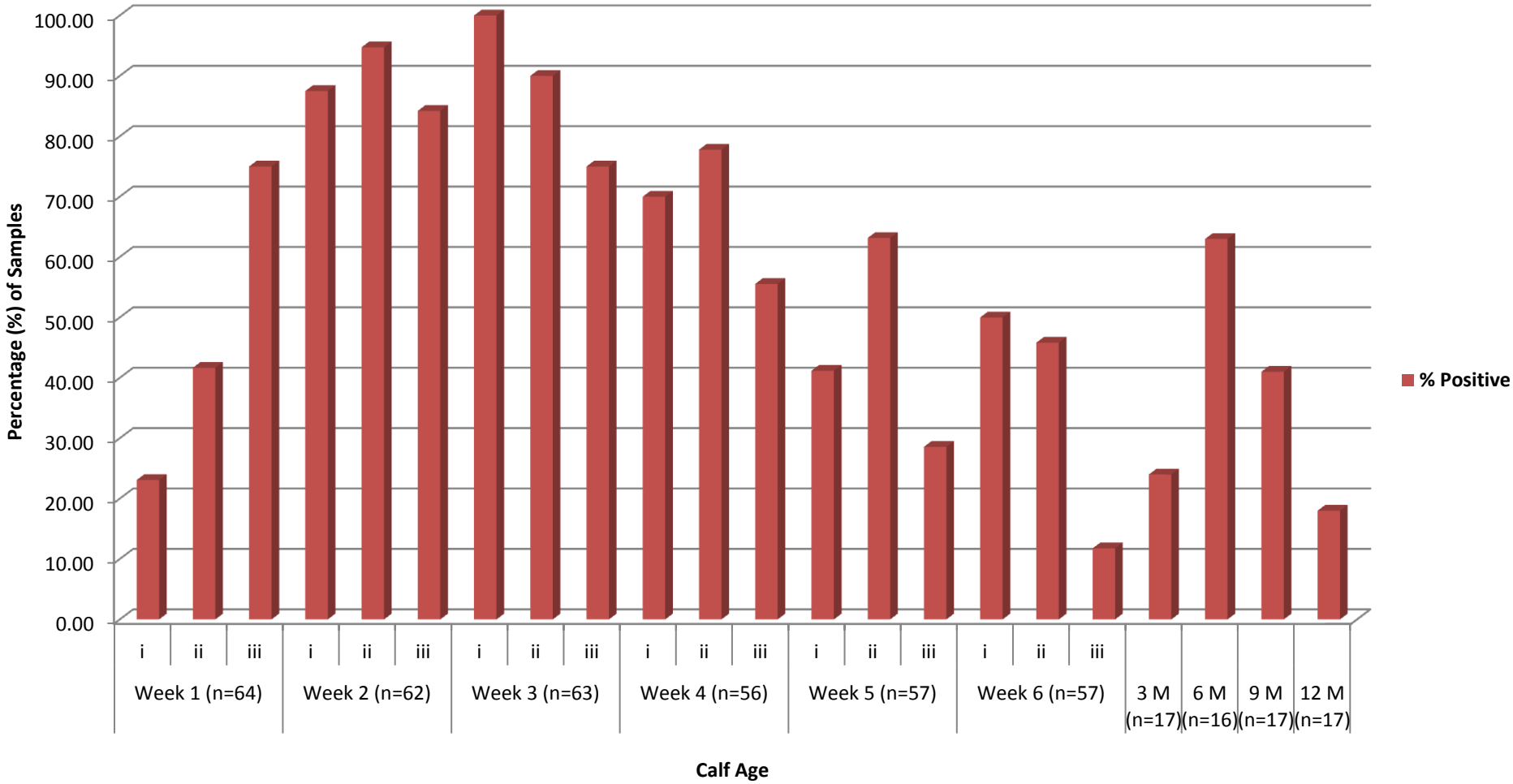


Figure 5-1: Shedding profile of *Cryptosporidium* oocysts in calves from birth to 12 months.
Percentage of positive samples from calves in the first six weeks of life and at 3, 6, 9 and 12 months of age. The percentage of positive samples at each sampling point (i, ii, iii) is indicated by a red bar.

	Calf Age (Weeks)																	Calf Age (Months)				
Calf ID	**Week 1**			**Week 2**			**Week 3**			**Week 4**			**Week 5**			**Week 6**			**3 M**	**6 M**	**9 M**	**12 M**	**% Positive**
2384	N	N	P	P	P	P	P	P	P	N	P	0	0	P	N	0	N	N	P	0	P	N	61.1
2385	N	P	P	P	P	N	0	P	P	P	P	0	0	P	P	0	P	N	N	N	N	N	61.1
2386	0	N	P	P	P	N	P	N	P	N	P	P	0	P	P	0	N	0	0	0	0		64.3
2387	0	N	P	P	P	N	0	P	P	P	P	P	0	P	P	0	N	0	0	0	0		76.9
2388	0	P	P	P	P	P	P	N	P	P	P	P	0	P	N	0	N	N	P	P	N	N	68.4
2389	N	N	P	N	N	P	0	P	0	P	P	0	N	N	0	P	0	0	0	0	0		50.0
2390	N	N	P	N	P	0	P	P	N	P	P	0	N	N	0	0	N	N	N	P	N	N	38.9
2393	N	P	N	P	P	0	P	P	P	P	0	0	N	0	N	N	P	N	N	P	P	N	55.6
2394	N	N	P	N	P	0	0	P	P	P	0	0	P	0	P	N	P	N	0	0	0		61.5
2395	N	N	N	P	0	P	P	P	P	P	0	N	N	0	N	P	P	N	0	0	0		53.3
2396	N	N	N	P	P	P	P	P	P	P	0	N	N	0	N	N	P	N	0	0	0		50.0
2397	0	P	P	P	P	P	P	P	P	0	0	N	0	P	0	P	P	P	N	P	P	0	87.5
2398	N	N	P	P	0	P	P	P	P	0	P	N	0	0	N	0	P	N	P	P	P	N	64.7
2399	0	0	N	P	P	P	P	0	N	0	N	P	0	P	N	P	N	N	0	0	0		53.8
2402	P	P	P	P	P	P	P	0	N	N	0	N	N	N	N	N	N	0	N	N	P	N	42.1
2403	P	P	P	P	P	P	P	0	P	N	0	0	N	P	N	0	N	N	P	P	P	N	66.7
2404	0	P	N	P	P	0	0	0	P	0	P	P	P	0	N	N	N	P	N	P	N	N	56.3
2405	0	N	N	P	P	P	P	0	P	P	P	P	P	P	N	P	N	N	N	P	N	N	60.0
2407	0	N	P	P	P	P	0	P	N	0	P	P	P	P	P	P	P	N	N	P	N	N	68.4
2409	0	P	0	0	P	0	P	P	P	P	N	N	N	N	N	N	N	N	0	0	0	0	42.9
2411	0	P	P	P	P	0	P	P	P	N	P	N	P	P	N	N	P	N	N	N	N	N	55.0
2412	P	N	P	P	0	P	P	P	N	P	N	P	P	P	0	P	P	0	N	P	P	N	73.7
2413	0	P	P	P	0	P	P	P	P	P	P	N	N	N	N	N	N	0	N	N	N	N	47.4
2415	N	N	P	P	0	P	P	P	P	N	P	P	N	N	N	0	N	0	N	N	N	P	47.4
2417	0	N	P	P	0	P	P	P	N	P	N	P	P	N	P	P	P	0	N	N	N	P	63.2
% Positive	23.1	41.7	75.0	87.5	94.7	84.2	100.0	90.0	75.0	70.0	77.8	55.6	41.2	63.2	28.6	50.0	45.8	11.8	23.5	62.5	41.2	12.5	

Figure 5-2: Positive and negative samples collected from each calf.
Samples collected from each calf at each time point. A positive sample is denoted by a red “P” while a negative sample is denoted by a blue “N”, “0” indicates that a sample was not collected from that calf at on that day. The percentage of positive samples at each time point are shown at the bottom of each column while percentage of positive samples from each individual is shown in the right-hand column.

5.4.2 Speciation of *Cryptosporidium*-positive samples by nssm-PCR

All *Cryptosporidium*-positive samples were speciated using a nssm-PCR (Chapter 4). Three species were identified (*C. parvum*, *C. bovis* and *C. ryanae*) in 228, 21 and 12 samples respectively. In four samples the species present could not be determined. Fourteen mixed infections were identified: these were *C. parvum* and *C. bovis* (n=7) and *C. bovis* and *C. ryanae* (n=7). In this study *C. parvum* was the predominant species detected in calves <6 weeks old with seven calves showing evidence of mixed infections. Mixed infections of *C. parvum* and *C. bovis* were detected between three and five weeks and *C. parvum* and *C. ryanae* were seen only in week 6. The species shed over the initial six week period and at the other age groups are shown in Figure 5-3.

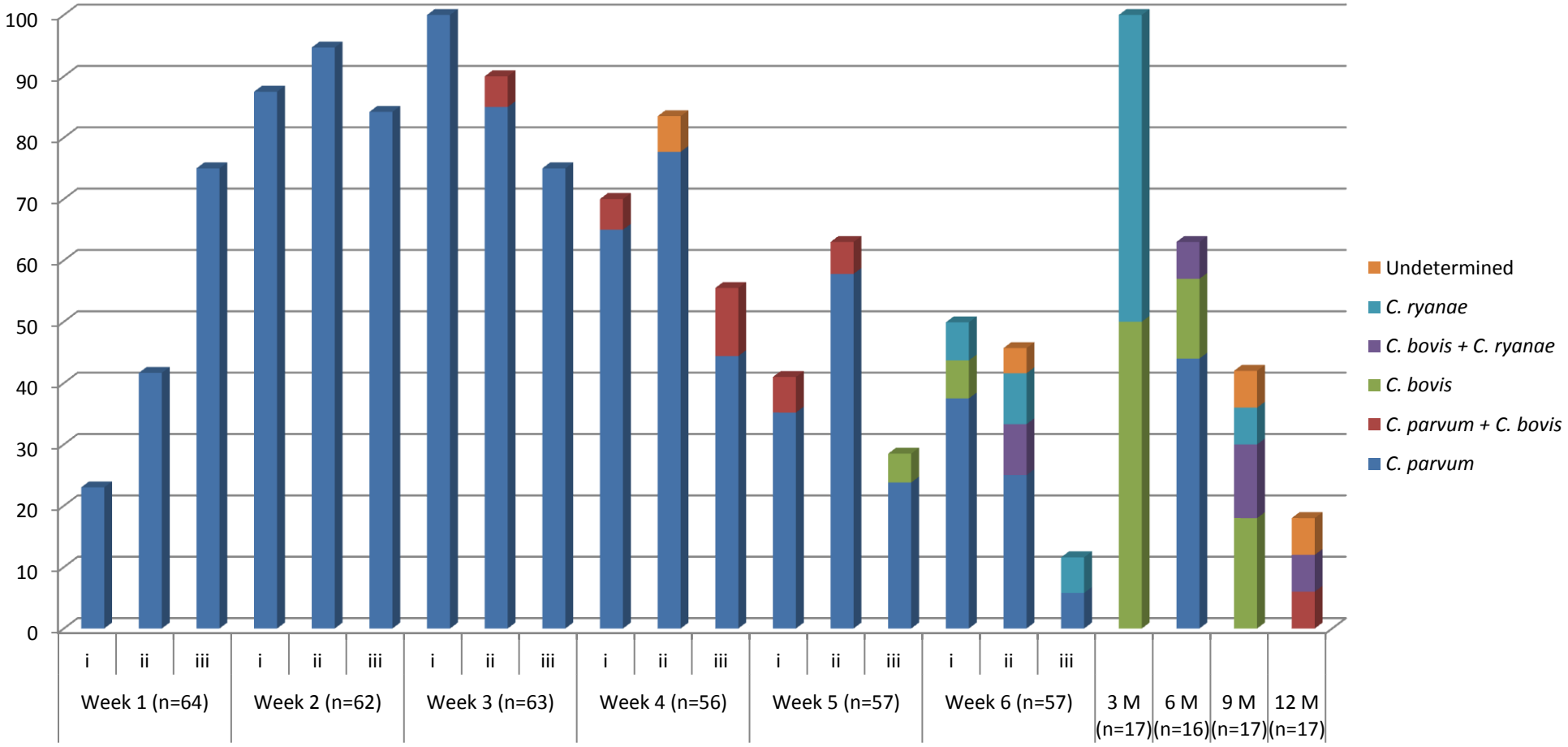


Figure 5-3: Prevalence of *Cryptosporidium* species in each age group of cattle.
Percentage of each species of *Cryptosporidium* at each time point.

To confirm the results of the nssm-PCR a selection of samples were sent for sequencing by GATC (see section 2.7) and all sequenced samples matched the result of the nssm-PCR.

5.4.3 Subtyping of *C. parvum* by *gp60* PCR

To determine the *gp*60 subtypes *C. parvum* positive samples were subjected to a nested PCR to amplify a fragment of the gp*60* gene. Two hundred and eight of these were positive, a sub-set of which, selected from 5 calves which shed *C. parvum* throughout the initial 6 weeks and at 6 months as well as others which were only *C. parvum* positive in the first 6 weeks, were sequenced (n=30). Two different *gp*60 subtypes were identified. In all of the samples from the calves <6 weeks the genotype identified was IIaA19G2R1 and in the samples from the same calves at 6 months the genotype identified was IIaA15G2R1 (Table 5-2).

Table 5-2: *gp60* genotypes detected in calves at different ages.

Calf ID	< 1 Weeks	2-3 Weeks	6 Weeks	6 Months
2384		IIaA19G2R1		
2385		IIaA19G2R1	IIaA19G2R1	
2388	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	IIAa15G2R1
2393	IIaA19G2R1	IIaA19G2R1		IIAa15G2R1
2395	IIaA19G2R1	IIaA19G2R1		
2397		IIaA19G2R1		IIaA15G2R1
2398	IIaA19G2R1	IIaA19G2R1		
2404	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	IIaA15G2R1
2405	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	IIaA15G2R1
2407	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	
2417	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	

5.5 Discussion

Although many prevalence studies (longitudinal and point-prevalence) investigating *Cryptosporidium* in cattle have been carried out worldwide this study is the most in-depth study carried out in the UK and provides detailed information on the species and subtypes of *Cryptosporidium* shed in a group of calves over a 12 month period. The findings of this study are comparable with those found by others investigating species of *Cryptosporidium* in calves of a similar age; Santin et al. (2004) found that *C. parvum* was the predominant species detected in calves <2 months with *C. bovis* and *C. ryanae* being detected in some samples from week 3 onwards, in another study *C. parvum* was the only species detected in calves <4 weeks with *C. bovis* being detected in two samples at week 4 and one at week 5. *Cryptosporidium ryanae* was not detected until week 10 (Santin et al., 2008). A more recent study from France (Rieux et al., 2013) also showed that *C. parvum* was the most prevalent species of *Cryptosporidium* in young calves (7-17 days old) with *C. bovis* first being detected at around week 2 and *C. ryanae* from week 3.

In the present study, when the same calves were examined at 3 months, only two species were detected and there were no mixed infections with 50% of the positive samples being identified as *C. bovis* and 50% as *C. ryanae*. These species are the most commonly detected in calves of this age group in other studies including longitudinal and point prevalence studies (Follet et al., 2011; Langkjaer et al., 2007; Ng et al., 2011; Santin et al., 2008; Santin et al., 2004; Silverlas et al., 2010). Few studies have examined calves older than three months and most show that the most commonly detected species in this age group (~6 months) are *C. bovis* and *C. ryanae* (Follet et al., 2011; Langkjaer et al., 2007; Santin et al., 2008; Santin et al., 2004; Silverlas et al., 2010) and occasionally *C. andersoni* (Santin et al., 2004), *C. ubiquitum* (Follet et al., 2011) or *C. suis*-like (Langkjaer et al., 2007). In the group of calves studied here we report a more unusual distribution of *Cryptosporidium* species for the age of cattle, at three months the calves shed only *C. bovis* and *C. ryanae* which is as expected however, in the same calves at 6 months old 70% of the positive samples were identified as *C. parvum* (by both nssm-PCR and sequencing of a fragment of the 18S rRNA gene), 20% as *C. bovis* and 10% as a mixed infection of *C. bovis* and *C. ryanae*. This is very unusual as *C. parvum* in 6 month old cattle

has only been reported a handful of times, and usually as a small percentage of positive samples.

In one study, which examined faeces from older calves *C. parvum* was detected in 5% of calves between the age of 31 days and 6 months (n=368) but it is not stated at exactly which age the calves were shedding *C. parvum* (Follet et al., 2011) and in another study from the USA (Santin et al., 2008) *C. parvum* was found in one calf at 6 months. In the present study, when the same calves were sampled at 9 months of age, no *C. parvum* positive samples were detected. Forty-three percent of the positive samples were identified as *C. bovis*, 14% *C. ryanae* and 29% as a mixed infection of *C. bovis* and *C. ryanae*. Again this ties in with other studies done on this age group of cattle (Langkjaer et al., 2007; Santin et al., 2008; Santin et al., 2004).

The first detection of *Cryptosporidium* positive samples occurred at day two of age in three calves with the majority of calves producing a positive sample between day four and six of life. This early detection indicates that calves pick up the parasite very soon after birth and while it might seem unlikely that calves can become infected and be shedding oocysts in such a short period of time the prepatent period of *Cryptosporidium* in calves is known to be 2-7 days (Tzipori et al., 1983). The first born calves started to shed oocysts at 5-7 days while later born calves began shedding between 2-4 days. This may be an indication that as calving progresses there is a build-up of environmental contamination which means later born calves receive a higher infective dose and therefore begin to shed oocysts sooner. It is known that the prepatent period for *Cryptosporidium* is also dependant on infective dose (Tzipori et al., 1983).

The previously reported age distribution of *Cryptosporidium* species in cattle has been confirmed again in this study with the majority of calves under 6 weeks of age being infected with *C. parvum*. However, in this study we also report the occurrence of *C. bovis* and *C. ryanae* in this age group, which is less common. We also show evidence of mixed infections within this age group and others (6, 9 and 12 months). It has been previously shown that even when using sensitive molecular methods to detect *Cryptosporidium* species in faecal samples, mixed infections can be missed because the predominant species is preferentially amplified (Cama et al., 2006). The detection method used in this study (nssm-

PCR) is much more effective at detecting mixed infections than standard 18S PCR, this accounts for the increased detection of mixed infections in these calves compared to similar studies. This is of particular use in terms of public health, when a low level of infection with the zoonotic species *C. parvum* is missed because of a predominant infection with a less pathogenic species such as *C. bovis* or *C. ryanae*.

Recent advances in molecular techniques have enabled further characterisation of some *Cryptosporidium* species, this can help provide further information and aid in the understanding of parasite transmission and pathogenicity. In the present study subtyping of *C. parvum* delivered some interesting and unusual findings. It has been demonstrated previously that most farms tend to have to have only a single *gp*60 subtype present (Broglia et al., 2008; Brook et al., 2009) (Santin et al., 2008) particularly where there is little movement of cattle. On the study farm replacement heifers are reared on the farm and the only animal bought in is a bull every couple of years. In contrast, on farms where there is greater cattle movement (buying in stock) more than one subtype may be found (Brook et al., 2009; Follet et al., 2011; Silverlas and Blanco-Penedo, 2013). On the farm in this study it appears that there are two distinct populations (one in the young calves and one in the older calves) despite the fact that very few animals are bought in. The subtype identified from the 6 month old calves is the most commonly detected *gp*60 subtype in cattle and humans worldwide and has been found in cattle in many different countries including; Belgium (Geurden et al., 2007), Great Britain (Brook et al., 2009; Thompson et al., 2007), Germany (Broglia et al., 2008), Italy (Duranti et al., 2009), The Netherlands (Wielinga et al., 2008), Portugal (Alves et al., 2006; Alves et al., 2003), Romania (Imre et al., 2011) and Spain (Quilez et al., 2008b). This subtype is extremely common and one review article reports that 55.3% of *C. parvum* subtypes in cattle were identified as IIAa15G2R1 (Imre and Darabus, 2011). In contrast, the subtype identified in the samples from the <6 week old calves (IIaA19G2R1) is much less common than IIAa15G2R1 in calves (Couto et al., 2013; Wielinga et al., 2008; Xiao et al., 2007) and humans (Gait et al., 2008; Hadfield et al., 2011). However, it is has been present on this particular farm for many years and is known to be zoonotic (Gait et al., 2008).

The management of cattle on this farm is very good: calves are kept in straw bedded pen in groups of ~20 from birth until they reach 3 months then the groups are mixed and moved to a new straw bedded shed (group sizes of ~40). Once the calves are older (5/6 months) they are moved again and all calves in the same age range are housed in a concrete floored pen with cubicles or turned out depending on the time of year. This could explain the different subtypes being found in different areas of the farm as different age groups of cattle are never mixed or housed in the same pens. It is also possible that there are two subtypes circulating in the calves but that one (IIaA19G2R1) is more pathogenic than the other (IIaA15G2R1) and that infection with this subtype overwhelms the gut and the second subtype is not seen until later in life. It has been demonstrated that in humans clinical disease and shedding of oocysts varies depending of species and subtype of *Cryptosporidium* (Cama et al., 2008) and the same may be true in cattle.

Although some longitudinal studies have been carried out in cattle, most have focused on pre-weaned animals or have been point prevalence studies only looking at a group of animals at one particular time point, this study has investigated the prevalence of *Cryptosporidium* species in a group of animals over a longer period with more frequent sample collections (3/week initially) providing more detailed information on how the prevalence of *Cryptosporidium* in a group of calves changes as the animals get older. Of the few studies which have looked at older calves many report that as the calves' age increases the prevalence of *Cryptosporidium* decreases (Follet et al., 2011; Rieux et al., 2013; Santin et al., 2008; Silverlas et al., 2010). This study reports that prevalence of *Cryptosporidium* decreases after 6 weeks with peak shedding at 3 weeks with a secondary peak in prevalence at 6 months. This pattern has been observed in two other studies, one in the USA and the other in The Netherlands (Huetink et al., 2001; Santin et al., 2004), the secondary peaks in shedding observed in the other studies were lower than reported here (~25% and 3.3% respectively), this is possibly due to the increased sensitivity of the techniques used in this study compared with previous studies.

5.6 Conclusion

It has been shown from this work that young calves become infected with, and begin shedding, *Cryptosporidium* very soon (minimum 2 days) after birth and that shedding of oocysts (with and without clinical disease) occurs for at least 6 weeks. It has also been demonstrated that calves can be infected with multiple species of *Cryptosporidium* simultaneously in the first 6 weeks of life. It was previously assumed that older post-weaned calves and adult cattle were not a significant risk for zoonotic transmission of *C. parvum* as most studies indicated that once calves were older they were not usually infected with this species. This study shows that even if calves are infected with *C. parvum* before weaning they can become infected with this species again, although a different subtype, when they are older. This is an important factor when considering the zoonotic implications, it also demonstrates the importance of molecular characterisation and speciation of all *Cryptosporidium* positive samples. Many older studies report "*C. parvum*-like" oocysts, since there are no morphological differences between *C. parvum*, *C. bovis* and *C. ryanae* this molecular characterisation is very important to make sure that the species is correctly and accurately assigned.

Further subtyping which was carried out in this study showed evidence that multiple subtypes of *C. parvum* may be present on a single farm and that these different subtypes may have different clinical manifestations, this is important from both an animal and public health perspective.

Finally, the techniques used in this study demonstrate the need to use the most sensitive methods available to detect oocysts and assign species particularly in the case of older animals where a predominant infection with a non-pathogenic species may be hiding a lesser infection with a pathogenic species such as *C. parvum*.

Papers in preparation and presentations at conferences

1. **Thomson, S.,** Wells, B., Harvey, C., Jonsson, N., Innes, E.A., Katzer, F., Species and genotypes of *Cryptosporidium* in calves from birth to 1 year. (In. Prep)
2. Species and genotypes of *Cryptosporidium* found in calves from birth to one year – **2nd International apiCOWplexa Conference** – Turkey, 2013
3. **Shedding of zoonotic *Cryptosporidium parvum* by dairy calves from birth to six months** – CoZee Conference – Scotland, 2012

Chapter 6: Transmission of *Cryptosporidium* from dam to calf

6.1 Introduction

Many different routes have been proposed for the transmission of *C. parvum* to calves (Figure 6-1) and one suggestion is that adult cattle may be the source of infection for neonatal calves. Calves tend to become infected with *Cryptosporidium* shortly after birth, within 24-72 h (Ramirez et al., 2004; Xiao and Herd, 1994) therefore a likely source for infection would be their dams as the calves are in close contact with them immediately after birth. A few studies have been carried out to test this hypothesis where the presence of *Cryptosporidium* in the adult faecal samples was detected using microscopy or ELISA (to detect *Cryptosporidium* antigens in faecal supernatant using an anti-*Cryptosporidium* antibody) and from this it was assumed that the *Cryptosporidium* in the adult animals were the source of infection for the neonatal animals. However, the methods used in these studies were unable to identify the species of *Cryptosporidium* only that there were oocysts present. Now that it is known that there are many different species of *Cryptosporidium* and that cattle may be infected with four main species, the question of the source of *C. parvum* infection for neonatal animals needs to be clarified.

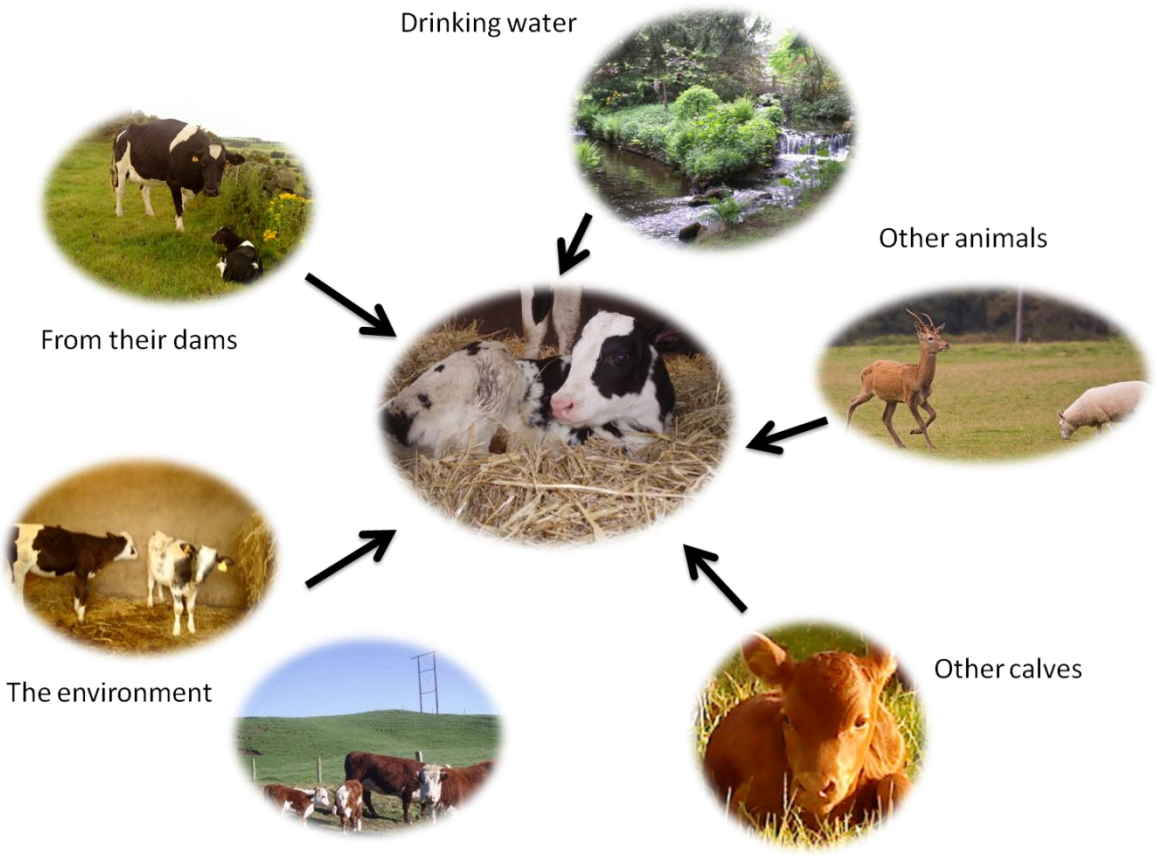


Figure 6-1: Possible routes for transmission of *Cryptosporidium* to calves.
Many different routes for transmission of *Cryptosporidium* to young neonatal livestock have been suggested, the most common of these are transmission from dam to calf at or shortly after birth, from the environment (cattle shed or contaminated pasture), direct contact with contaminated faeces of another host or from contaminated drinking water supplies.

In adult cattle excretion of oocysts tends to be low and intermittent and there are studies to demonstrate this (Table 6-4). One reason for this may be that the methods used are not sensitive enough to detect oocysts in adult cattle faeces. Studies on sheep suggested that dams were the likely source of infection to their young due to a periparturient rise in oocyst excretion (Ortega-Mora et al., 1999; Xiao et al., 1994) leading to the suggestion that the same may be true of cattle. However this evidence was based on the assumption that the oocysts shed were *C. parvum* and at this time many of the other species which we now know infect farm livestock had not been identified. More recent studies show no evidence for a periparturient rise in oocyst shedding in cattle (Atwill and Pereira, 2003; Sturdee et al., 2003) and dams as a source of infection for calves were suggested to be of minor importance (De Waele et al., 2012) except for the transmission of *C. bovis* in older calves (Kvac et al., 2011). Another study (Faubert and Litvinsky, 2000) showed that a periparturient rise in oocyst shedding in cattle does occur and that cows are a likely source of infection for their calves.

A more probable source of infection for young calves is environmental contamination (from other calves or hosts); *Cryptosporidium* oocysts are highly resistant to heat, cold, disinfectants and are very difficult to eliminate (Robertson et al., 1992). It is thought that differences in calf management practices such as housing can have an effect on infection rates but some of the results are conflicting. In one study of 2024 calves from 248 farms over a two year period, it was shown that housing calves with their dams decreased the risk of *C. parvum* infection while calves removed from their dam and kept in individual pens were at higher risk of infection (Duranti et al., 2009) hinting that dam to calf transmission is less important than calf to calf or via environmental transmission. In contrast, other studies have shown that the opposite is true (Quigley, III et al., 1994; Quigley, III et al., 1995) or that calf housing (individual or group penning), has no effect on *Cryptosporidium* infection (Castro-Hermida et al., 2002) though calves housed on straw rather than concrete were at higher risk of infection regardless of whether or not they were housed individually or in a group. It is also possible for infection to be passed between calves in a particular pen (O'Handley et al., 1999).

One reason for the apparent low level or lack of shedding of *Cryptosporidium* oocysts by adult animals (cattle and sheep) may be due to the lack of sensitivity of the methods used in processing the faecal samples. Faecal samples from adult animals, particularly cattle are much larger in volume than those from calves and contain more fibrous material meaning that detection of oocysts is much harder. In many studies when negative samples have been detected it is possible that these samples are actually positive but have been missed by the techniques used. In the first part of this study several techniques were assessed to determine the most effective method of processing adult cattle faeces.

6.2 Aims

1. Develop a more sensitive method for oocyst concentration and extraction from adult cattle faeces

2. Assess the shedding profile of different *Cryptosporidium* species found in dams throughout gestation and after calving

3. Determine the *Cryptosporidium* species found in the calves born to cows in the study

4. Determine the subtypes of *C. parvum* in the same dams and calves

6.3 Materials and Methods

6.3.1 Optimisation of oocyst concentration and DNA extraction from adult cattle faeces

6.3.1.1 Spiking Experiment

A *Cryptosporidium* negative faecal sample (as determined by 10 × DNA extractions and PCR) was obtained from a one year old Charolais bull from the University of Glasgow, School of Veterinary Medicine. The animal was born at the veterinary school and had never had access to grazing and had limited access to other cattle and livestock. To test each oocyst concentration method (direct DNA extraction, acid flocculation (AF), salt flotation (SF) and combined acid flocculation and salt flotation) 0, 5, 10, 100 and 1000 *C. parvum* oocysts (obtained from experimentally infected calves) per gram were added to each sample as shown in Table 6-1.

Table 6-1: Methods tested for optimisation of oocyst concentration before DNA extraction from cattle faeces.
Number of oocysts spiked per gram of faeces and volume of faeces used for each method.

	Method			
No. of oocysts per gram	Acid Flocculation	Salt Flotation	Acid Flocculation and Salt Flotation	No Prior Concentration
0	50 g	3 g	50 g	250 mg
5	50 g	3 g	50 g	250 mg
10	50 g	3 g	50 g	250 mg
100	50 g	3 g	50 g	250 mg
1000	50 g	3 g	50 g	250 mg

6.3.1.2 Field Samples

Using faecal samples (n=205) collected from adult dairy cattle as part of this study, AF followed by DNA extraction was compared with AF/SF followed by DNA extraction.

6.3.1.3 Acid Flocculation

The faecal sample was well mixed and 50 g added to a 1 L cylinder with 600 ml water and 7 ml 2% sulphuric acid. The sample was put on a magnetic stirrer and thoroughly mixed for 5 mins then left to settle for approximately 5 mins or until

a clear line was visible between the sediment and supernatant, after which the supernatant was removed by pipette, collected in two 250 ml bottles and centrifuged for 20 mins at 1000 *x g*. The supernatant was discarded and 6 ml H_20 was added to the pellet, mixed and put into a 15 ml tube, which was centrifuged for 5 minutes at 3000 *x g*. The supernatant was discarded and the pellet retained for further processing by either DNA extraction (6.3.1.5) or salt flotation (6.3.1.4).

6.3.1.4 Salt Flotation

A 3 g faecal sample or pellet from acid flocculation was added to 8 ml saturated salt solution and thoroughly mixed by vortexing and 2 ml dH_2O trickled on top of the salt solution before centrifugation at 1000 *x g* for 8 mins. Following centrifugation the water layer was gently swirled using a Pasteur pipette to create a vortex which draws the oocysts from the layer between the dH_2O and salt into the dH_2O, the layer was removed and added to 6 ml dH_2O, the total volume was made up to 10 ml, mixed by inverting and centrifuged at 5000 *x g* for 5 mins. The supernatant was poured off and discarded and the pellet retained for DNA extraction (Ryley et al., 1976).

6.3.1.5 DNA Extraction

DNA was extracted as described in section 2.5.1.

6.3.1.6 PCR amplification of the 18S rRNA gene

DNA samples were amplified using the 18S PCR described in section 2.6.1 and visualised on a 1.5% agarose gel stained with GelRed (Biotium, UK). All reactions were carried out in triplicate and a sample was considered positive if a band corresponding with ~840 bp was seen. Several controls, both negative (no template) and positive (*Cryptosporidium* DNA) were run with each set of reactions. A positive result was only accepted when all negative controls were negative and positive controls were positive.

6.3.2 Sample Collection

6.3.2.1 Adults

The study farm used for this study was the same farm which was used for the study described in Chapter 5. Faecal samples were collected from 29 in-calf adult dairy cattle (heifers and cows) three times per week for up to ten weeks pre-calving, in total 205 samples were collected. The cows were kept under the normal working conditions of this farm and as such were housed in cubicle sheds before calving, in straw courts at calving and in slatted cubicle sheds after calving when they returned to the milking herd. Cows were observed in the cattle shed until they defecated and then the entire motion was collected in a plastic bag. Due to the method of collection it was not always possible to collect a sample from each cow at each sampling point. After collection the samples were transferred to the laboratory where the entire motion was mixed and a sub-sample aliquoted into a 125 ml tub and stored at 4°C.

6.3.2.2 Calves

Samples from calves (n=30) born to these cows were collected from the day of birth until 6-9 weeks of age three times per week (Monday, Wednesday and Friday). The samples were collected from the rectum of calves using a gloved finger and bag for collection, where possible the entire motion was collected. It was not always possible to collect a sample from each calf on each sampling day; therefore the number of samples collected per calf did vary. The calves were kept under the normal working conditions of this farm with regards to housing, feeding and veterinary treatment. The calves were kept with their mothers for one/two days then moved to a small group (5-8 calves) for one week then moved to a larger indoor straw bedded pen with approximately 20 calves in each pen for the remaining five weeks. After this time the calves were moved, and groups mixed, to a different, indoor straw bedded pen. Samples were collected from the same calves again at six months of age where possible. The total number of samples collected was 380. The samples were mixed thoroughly and aliquoted into bijoux tubes and stored at -20°C for further processing

6.3.3 Sample processing and DNA isolation

6.3.3.1 Adults

Samples were processed to concentrate *Cryptosporidium* oocysts from a 50 g starting sample by acid flocculation and salt flotation as described in section 2.4.1. DNA was isolated as described in section 2.5.1.

6.3.3.2 Calves

Faecal samples from calves were processed and DNA isolated as described in section 2.5.2.

6.3.4 Species identification by nssm-PCR (nested species specific multiplex-PCR)

For the differentiation of *Cryptosporidium* species an nssm-PCR was carried out using the first round PCR product from the 18S PCR (see section 2.6.1) as a template. The PCR products were visualised on a 1.5% agarose gel stained with GelRed (Biotium, UK). All reactions were carried out in triplicate and a sample was considered positive if a band corresponding with 840 bp and any combination of 625 bp, 415 bp, 305 bp or 241 bp was seen. Positive controls for each *Cryptosporidium* species tested for were included as well as extraction controls and no template controls. A positive result was only accepted when all negative controls were negative and all positive controls were positive.

6.3.5 *C. parvum* subtyping

Sequencing of *C. parvum* positive samples was carried out by amplification and sequencing of the *gp60* gene (see sections 2.6.3 and 2.7). The PCR products were visualised on a 1.5% agarose gel stained with GelRed (Biotium, UK). Reactions were originally carried out in duplicate and if amplification was not successful the reactions were repeated. A sample was considered positive if a band corresponding with ~375 bp was seen. Positive, negative and extraction controls were included for each set of reactions. A positive result was only accepted when all negative controls were negative and all positive controls were positive.

gp60 positive samples were sequenced in the forward and reverse orientation by GATC Biotech (GATC, Germany) as described for “supremerun96” sequencing in section 2.7.3. The sequences were analysed using Chromas Lite (version 2.01 Technelysium) and the subtypes named as according to (Sulaiman et al., 2005).

6.4 Results

6.4.1 Optimisation of oocyst concentration and DNA extraction from adult cattle faeces

6.4.1.1 Spiking Experiment

The results of the spiking experiment comparing the four methods of oocyst extraction from adult cattle faeces are given in Table 6-2 and Figure 6-2. Acid flocculation alone failed to concentrate oocysts; SF alone was sensitive to 100 oocysts/g; the combined AF/SF method to 5 oocysts/g and direct DNA extraction was sensitive to 100 oocysts/g of faeces. The PCR reactions were carried out in triplicate.

Table 6-2: Detection limits of each of the four concentration methods.

	Method			
No. of oocysts per gram	Acid Flocculation	Salt Flotation	Acid Flocculation and Salt Flotation	No Prior Concentration
0	-	-	-	-
5	-	-	++	-
10	-	-	+++	-
100	-	++	+++	+
1000	-	+++	+++	+++

- = negative, + = positive in 1 of 3 reactions, ++ = positive in 2 of 3, +++ = positive in 3 of 3 PCR reactions

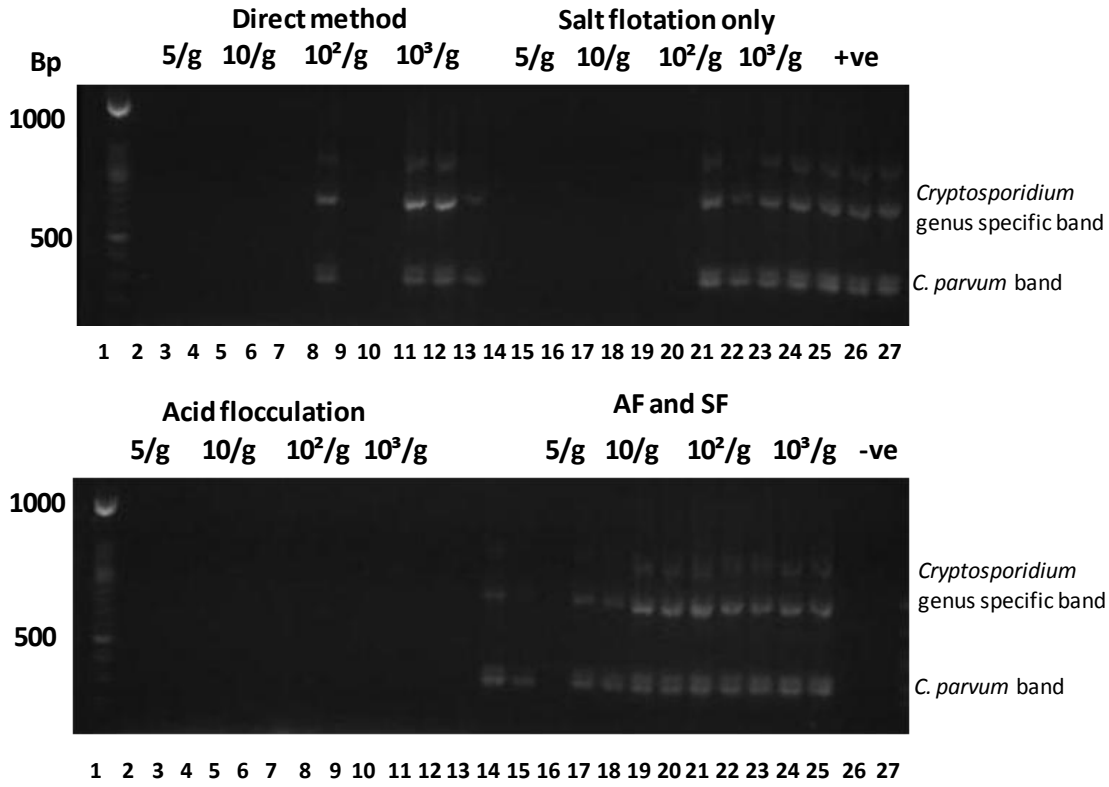


Figure 6-2: Spiking experiment to compare AF, SF, AF/SF and no concentration methods of oocyst detection from adult cattle faeces.
Top image: lane 1= DNA Molecular Weight Marker XIV 100 base pair ladder (Roche); lanes 2-13= direct method: 2-4=5 oocysts/g; 5-7=10 oocysts/g; 8-10=10^2 oocysts/g; 11-13=10^3 oocysts/g. Lanes 14-25=salt flotation only: 14-16=5 oocysts/g; 17-19=10 oocysts/g; 20-22=10^2 oocysts/g; 23-25=10^3 oocysts/g. Lanes 26-27= positive controls.

Bottom image**:** lane 1= marker (Roche 100bp); lanes 2-13= acid flocculation only: 2-4=5 oocysts/g; 5-7=10 oocysts/g; 8-10=10^2 oocysts/g; 11-13=10^3 oocysts/g. Lanes 14-25=acid flocculation and salt flotation: 14-16=5 oocysts/g; 17-19=10 oocysts/g; 20-22=10^2 oocysts/g; 23-25=10^3 oocysts/g. Lanes 26-27= negative controls.

6.4.1.2 Evaluation of AF and AF/SF

Two hundred and five faecal samples were tested for the presence of *Cryptosporidium* DNA by either AF followed by DNA extraction or AF/SF followed by DNA extraction. Of the 205, 11 tested positive using the acid flocculation method only and 57 tested positive using the AF/SF.

6.4.2 Prevalence of *Cryptosporidium*

6.4.2.1 Adult Cattle

In total 205 faecal samples from 29 adult dairy cattle (cows and heifers) were collected. On average seven samples from each animal were collected over the pre-calving period (range 3-13, median 7, mode 7). Due to the nature of

collection it was not possible to collect a sample from each animal every day, it was hoped that a minimum of one sample per cow per week would be obtained. The majority of samples were collected during the four week period immediately prior to calving (Figure 6-3).

	Pre-Calving																										Post-Calving						
I.D	**Week 9**			**Week 8**			**Week 7**			**Week 6**			**Week 5**			**Week 4**			**Week 3**			**Week 2**			**Week 1**			**Calving**	**Week 1**			**Week 2**			**Samples Per Cow**
254																		0	0	0	0	X	0	X	0	0	X	X							4
220													0	0	X	0	0	0	X	0	X	X	X	0	X	0	0	0	0	X	0	0	0	X	8
136														0	0	0	0	X	0	0	X	X	X	X	0	0	X	0	X						7
278													0	0	0	0	0	0	0	0	X	X	0	X	0	0	0	0							3
350													0	0	0	0	0	0	0	X	X	0	X	0	0	X	0	0							4
26												0	0	0	0	0	0	X	0	X	X	X	X	X	0	0	0	0	X						7
354												0	0	0	0	X	0	0	0	0	X	X	0	0	0	0	X	0							4
52											0	0	0	0	0	0	0	X	0	0	X	0	0	0	X	0	0	0	0	0	0	0	X		4
15											0	0	X	0	0	0	X	0	X	X	X	0	0	0	0	0	0	0	X						6
256											0	0	0	0	0	0	0	X	X	X	0	0	0	0	X	0	0	X	X						6
243										0	0	0	0	0	0	X	X	0	X	X	X	X	0	0	X	0	0	0							7
96										0	0	0	0	0	0	0	X	X	0	X	0	0	0	X	0	0	X	0							5
178										0	0	X	0	X	0	0	0	X	0	X	0	X	X	0	X	0	0	X							8
51										0	0	X	0	X	0	0	X	0	X	X	0	0	X	X	0	0	0	0							7
64										0	0	0	0	0	0	0	X	X	X	X	0	X	X	0	0	0	0	X							7
20								0	0	0	0	0	0	0	0	X	X	0	0	0	X	0	X	0	X	0	0	X	X							7
130								0	0	0	0	0	0	0	0	X	0	X	0	X	X	X	X	0	X	0	0	0	X		X					9
105								0	0	0	0	0	0	0	X	X	0	0	0	X	0	X	X	0	X	0	0	0							6
108							0	0	0	0	0	0	X	X	X	0	X	0	X	0	X	0	X	X	X	0	0	0	X	X						11
62						0	0	0	0	0	0	0	0	0	0	0	0	0	X	0	0	0	X	0	X	0	X	0							4
176						0	0	0	0	0	0	X	0	0	0	0	X	0	0	0	0	0	X	0	0	0	X	0	X							5
138					0	0	0	0	0	0	0	0	0	0	0	X	X	0	0	X	0	X	0	0	0	0	0	0	X	X						6
50					0	0	0	0	X	0	0	X	0	X	0	0	X	0	0	X	X	X	X	X	0	X	X	0	X	X						13
54				0	0	0	0	0	0	0	0	X	X	0	X	X	X	X	0	0	0	X	0	0	0	0	0	0	X							8
357		0	0	0	0	0	0	X	0	0	X	0	0	0	X	0	0	0	0	X	X	0	0	X	X	X	X	0	X							10
149	0	0	0	0	X	0	0	X	0	X	X	X	0	0	X	0	0	X	X	0	0	0	X	0	0	0	X	X								11
57	0	0	0	X	0	0	X	0	0	0	0	0	0	X	0	0	0	0	0	0	X	0	X	X	0	X	0	0							7
82	0	X	0	0	0	0	X	X	0	0	0	0	X	0	0	0	X	X	X	0	0	0	X	X	X	X	0	0							11
233	X	0	0	0	0	X	0	0	0	0	0	X	0	0	0	0	X	X	X	0	X	0	0	0	X	0	X	0	X							10
Total	1	1	0	1	1	1	2	3	1	1	2	7	4	5	6	7	13	12	11	14	16	14	17	11	13	5	10	6	13	4	1	0	1	1	**205**

Figure 6-3: Samples collected from each cow pre and post calving.
Faecal samples collected over the study period from 29 adult dairy cattle (cows and heifers). A blue "X" denotes a sample collected on that day while "0" shows that no sample was collected from that cow on that particular day. The total number of samples collected at each collection point are shown at the bottom of the figure and total number of samples collected from each cow are shown in the right-hand column.

All samples were tested for the presence of *Cryptosporidium* DNA by 18S nssm-PCR after oocyst concentration by acid flocculation and salt flotation. Overall 27.9% (n=57) of samples from adult cows tested positive for *Cryptosporidium*. Four cows did not test positive for *Cryptosporidium* at any point throughout the study, the remaining 25 cows tested positive on at least one occasion and two cows that tested negative for *Cryptosporidium* pre-calving tested positive post-calving. The percentage of *Cryptosporidium* positive samples remained fairly stable throughout the study period (Figure 6-4) at ~30%. There were more fluctuations when looking at each individual time point when the percentage of positive samples ranged from 0-100%.

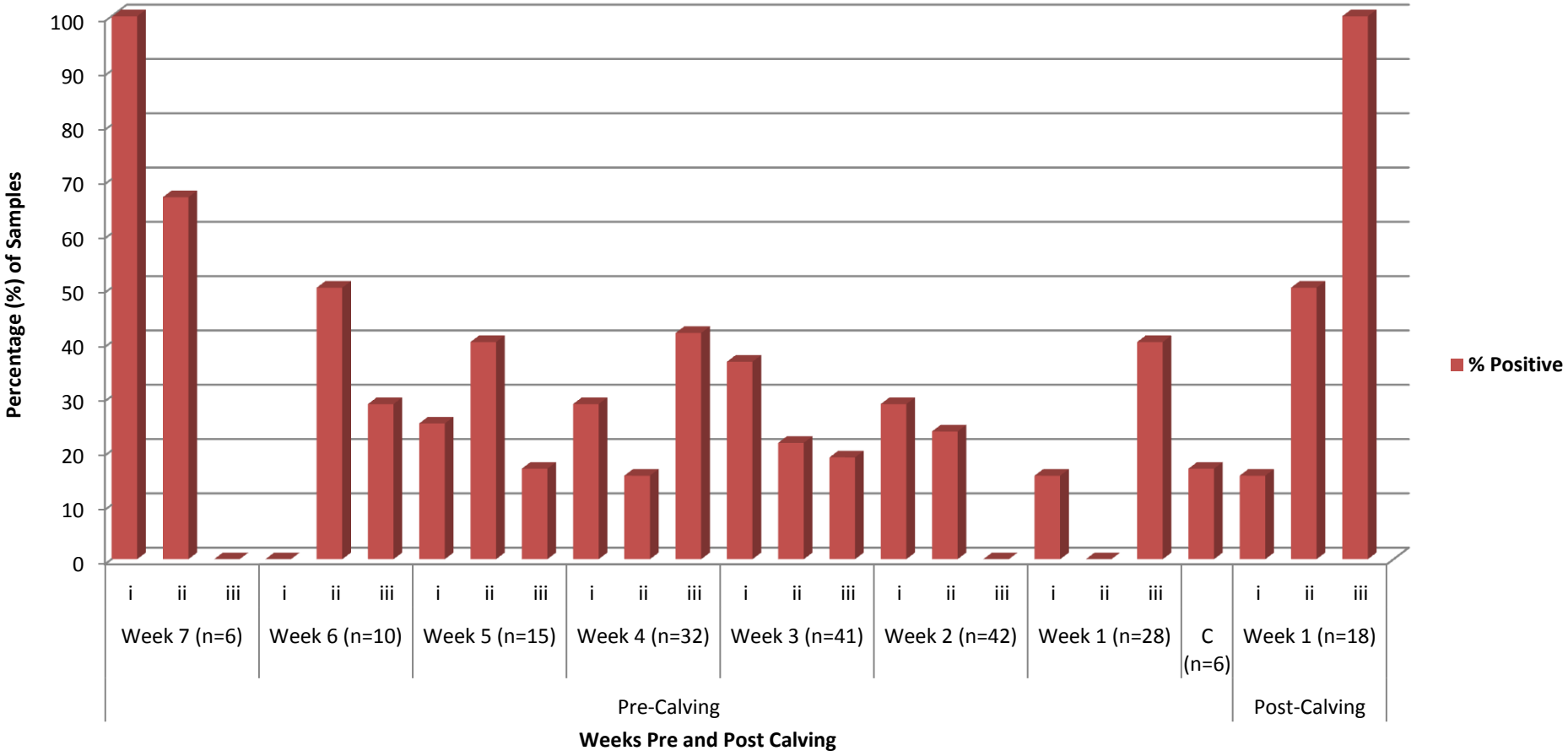


Figure 6-4: Positive samples detected in adult cows pre- and post-calving.
Percentage of positive samples detected in cows in the weeks pre- and post-calving. The percentage of positive samples at each sampling point (i, ii, iii) is indicated by a red bar. Weeks 8 and 9 pre-calving and week 2 post-calving have been omitted from this graph as only two samples were collected in each of these week. “C” indicates the day of calving, it was not possible to collect a sample from every cow on the day it calved.

6.4.2.2 Calves

In total 380 samples were collected, 364 from the initial collection period then 16 from the same calves at 6 months of age. On average 13 samples were collected from each calf (range 5-20, median 13, mode 14). Due to the nature of collection it was not possible to collect a sample from each animal every day, (Figure 6-5).

	Calf Age																										
Calf ID	**Week 1**			**Week 2**			**Week 3**			**Week 4**			**Week 5**			**Week 6**			**Week 7**			**Week 8**			**Week 9**			**6 Months**	**Total**
2596	0	0	X	0	X	0	X	0	X	0	X	X	X	0	X	0	0	X	0	X	0	X	0	X	0	X	X	0	14
2604	0	0	0	X	X	0	0	0	X	X	0	X	X	X	X	0	0	0	X	X	X	0	X	X	0	0	0	0	13
2605	0	X	0	X	X	X	X	X	X	X	0	X	X	X	X	0	X	0	X	X	X	0	X	X	X	X	0	0	20
2606	0	0	X	X	X	0	X	X	X	0	X	X	0	0	0	X	0	X	X	X	0	X	X	X	X	X	0	X	18
2607	0	0	X	X	X	X	X	X	X	0	X	X	X	X	0	X	0	0	X	X	0	0	X	X	X	0	0	0	17
2608	0	X	X	X	X	X	X	X	0	X	X	0	X	0	0	0	X	X	0	0	X	0	0	X	X		0	X	16
2609	0	0	0	X	X	X	X	X	0	X	X	X	X	0	0	0	X	X	0	0	X	0	X	X	0		0	X	15
2610	X	0	X	X	0	X	X	0	X	X	X	X	0	0	0	0	0	X	0	0	X	X	X	0				X	14
2611	0	0	0	X	X	0	X	X	0	X	X	0	X	0	X	0	X	X	0	0	X	X	X	X	X			0	15
2612	0	0	X	0	X	X	0	X	0	X	X	0	X	0	0	0	X	X	0	0	X	0	0	X	0			X	12
2613	0	0	X	X	0	X	X	0	X	X	X	X	0	X	0	X	X	X	0	X	0	0	0	0				0	13
2614	0	0	X	X	X	X	0	X	X	X	0	0	X	0	X	X	X	0	X	X	X	0	X					X	16
2615	0	0	X	X	X	X	0	X	0	X	0	0	X	0	X	X	X	0	X	0	X	X	0					X	14
2616	0	0	X	0	X	X	X	X	X	X	X	0	X	0	X	0	X	0	X	0	0	X	0					X	14
2617	0	0	X	X	X	X	X	X	X	X	X	0	X	0	X	X	X	0	0	0	X	X	X					X	17
2618	0	0	0	X	X	X	X	X	X	X	X	0	X	0	X	0	X	0	X	X	X	0	0					X	15
2619	0	0	0	X	X	X	X	X	X	X	X	0	X	0	0	X	X	0	0	0	X	0	0					X	13
2620	0	0	0	0	X	X	X	X	X	X	X	0	X	0	0	0	X	0	X	0	0	X	0					X	12
2621	0	0	X	X	X	X	X	X	0	X	0	X	X	X	0	X	0	0	0	0								0	11
2622	0	0	X	0	X	X	X	X	0	0	0	X	X	0	0	X	X	X	X	X								0	12
2623	0	X	0	X	X	X	X	X	0	X	0	X	X	X	0	X	0	X	X	X								0	14
2624	0	0	0	X	X	X	X	0	0	0	0	X	X	0	0	0	X	X	X									0	9
2625	0	0	0	X	X	X	X	0	X	0	X	X	0	0	0	X	X	X	0									X	11
2626	0	0	X	X	X	X	0	X	0	X	0	X	0	X	0	X	X	0										X	11
2627	0	0	X	0	0	X	0	X	X	X	0	X	X	X	X	0												X	10
2628	0	0	X	0	0	0	X	X	X	0	0	X	X	X	0													0	7
2629	0	0	0	X	0	X	X	X	0	X	X	X	X	X														0	9
2630	0	0	0	0	X	X	X	0	0	0	X	0	X															0	5
2631	0	0	X	0	X	0	X	X	X	X	0	0	0	0														X	7
2633	0	0	X	0	X	0	X	X	X	X	0	0	0	0														0	6
Total	1	3	18	20	25	23	24	23	18	22	17	17	23	10	10	12	17	12	12	10	12	8	9	9	5	3	1	16	**380**

Figure 6-5: Number of samples collected from each calf.

Faecal samples collected over the study period from 30 dairy calves from birth. A blue "X" denotes a sample collected on that day while "0" shows that no sample was collected from that calf at that particular collection point. The total number of samples collected at each collection point are shown at the bottom of the figure and total number of samples collected from each calf are shown in the right-hand column

All 380 samples were tested for the presence of *Cryptosporidium* parasite DNA using the 18S genus specific PCR and overall percentage of positive samples over the whole study was 51.0% (n=194). In the first nine weeks of life each calf tested positive for *Cryptosporidium* on at least 2 occasions, giving a period prevalence of 100% and the overall percentage of samples positive in calves <9 weeks was 51.9% (n=189). The earliest evidence of oocyst shedding occurred in one calf one day after birth, the majority of calves began shedding oocysts at day 4 (7 calves) or day 5 (6 calves) after birth. Peak shedding of *Cryptosporidium* oocysts occurred in week two with 95% of calves tested shedding oocysts (Figure 6-6). The percentage of positive samples per calf ranged from 22.2% to 80.0% although the calf with 80.0% positive samples did have fewer samples collected overall (Figure 6-7).

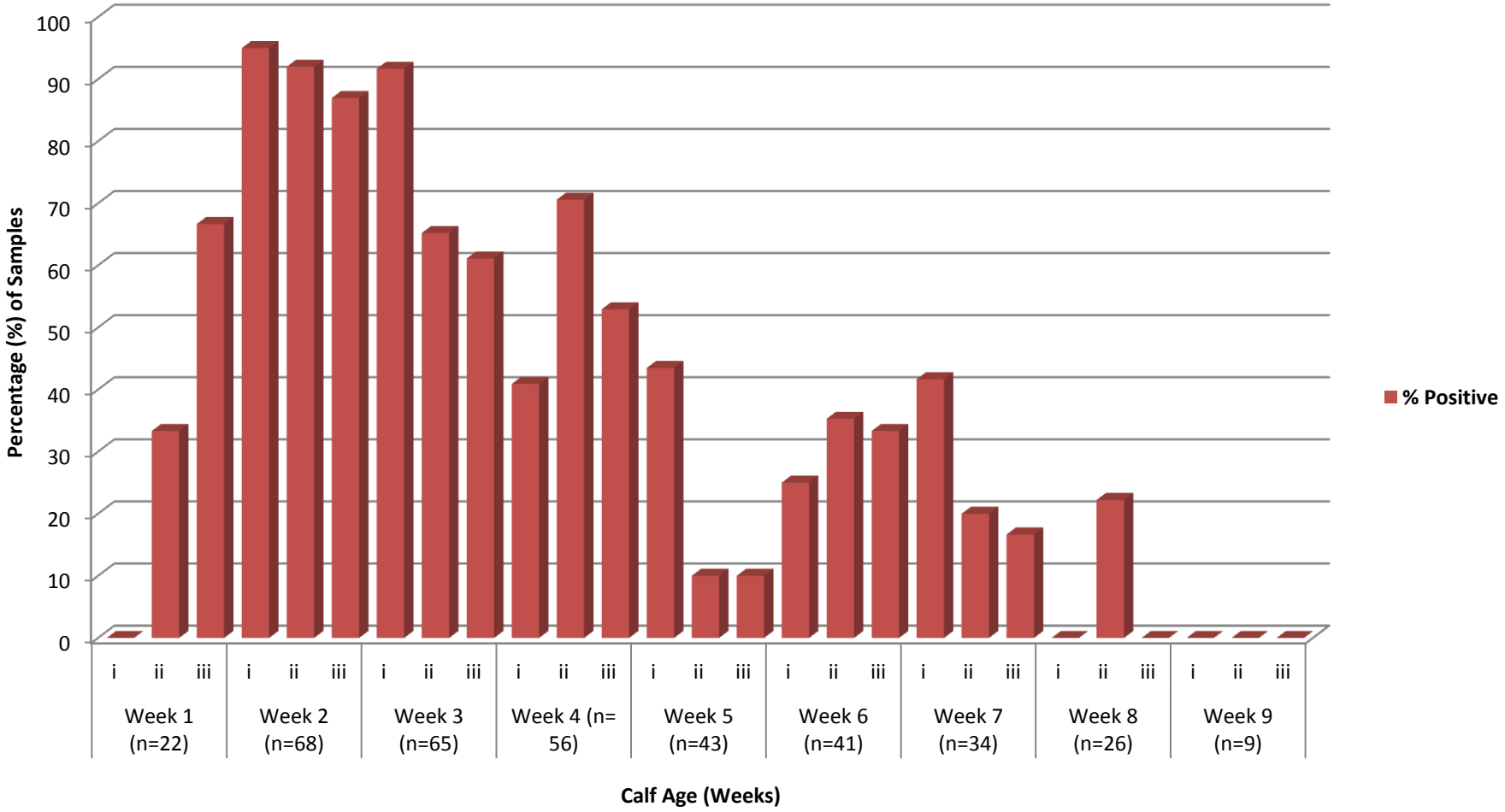


Figure 6-6: Shedding profile of *Cryptosporidium* oocysts in calves <9 weeks.
Percentage of positive samples detected in calves in the first nine weeks of life. The percentage of positive samples at each sampling point (i, ii, iii) is indicated by a red bar. Peak shedding of oocysts occurred in week 2 of life and tailed off towards the end of week 8/9.

	Calf Age																											
Calf ID	**Week 1**			**Week 2**			**Week 3**			**Week 4**			**Week 5**			**Week 6**			**Week 7**			**Week 8**			**Week 9**			**6 Months**	**% Positive**
2596	0	0	N	0	P	0	N	0	N	0	P	N	P	0	N	0	0	P	0	N	0	N	0	N	0	N	N	0	28.6
2604	0	0	0	P	P	0	0	0	P	N	0	P	P	N	N	0	0	0	N	N	N	0	N	N	0	0	N	0	35.7
2605	0	N	0	P	P	P	P	N	N	N	0	N	P	N	P	0	P	0	N	P	N	0	N	N	N	N	N	0	38.1
2606	0	0	P	P	P	0	P	P	N	0	P	P	0	0	0	P	0	N	P	N	0	N	N	N	N	N		N	50.0
2607	0	0	N	P	P	P	N	P	N	0	N	P	N	N	0	N	0	0	P	N	0	0	P	N	N	0		0	41.2
2608	0	N	P	P	P	P	P	N	0	P	P	0	P	0	0	0	N	N	0	0	P	0	0	N	N			N	56.3
2609	0	0	0	P	P	P	P	P	0	P	P	P	N	0	0	0	N	P	0	0	N	0	P	N	0			P	73.3
2610	N	0	P	P	0	N	P	0	P	N	N	P	0	0	0	0	0	N	0	0	P	N	N	0				N	42.9
2611	0	0	0	P	P	0	P	P	0	N	P	0	P	0	N	0	N	P	0	0	N	N	N	N	N			0	46.7
2612	0	0	P	0	N	P	0	P	0	N	P	0	P	0	0	0	N	N	0	0	N	0	0	N	0			P	50.0
2613	0	0	P	P	0	P	P	0	P	P	P	N	0	P	0	N	P	N	0	N	0	0	0	0				0	69.2
2614	0	0	P	P	P	P	0	N	P	N	0	0	N	0	N	P	N	0	N	P	N	0	N					N	43.8
2615	0	0	P	P	P	P	0	P	0	N	0	0	N	0	N	N	N	0	N	0	N	N	0					N	35.7
2616	0	0	N	0	P	P	P	N	P	N	P	0	P	0	N	0	N	0	N	0	0	N	0					N	42.9
2617	0	0	P	P	P	P	P	N	P	N	P	0	P	0	N	N	N	0	0	0	N	N	N					N	47.1
2618	0	0	0	P	P	N	P	P	P	P	N	0	N	0	N	0	N	0	P	N	N	0	0					P	53.3
2619	0	0	0	P	P	P	P	P	P	P	P	0	P	0	0	N	N	0	0	0	N	0	0					N	69.2
2620	0	0	0	0	P	P	P	P	P	P	P	0	N	0	0	0	N	0	P	0	0	N	0					P	75.0
2621	0	0	P	P	P	N	P	P	0	P	0	N	N	N	0	N	0	0	0	0								0	54.5
2622	0	0	N	0	P	P	P	P	0	0	0	N	N	0	0	P	P	N	N	N								0	50.0
2623	0	P	0	P	P	P	P	P	0	N	0	N	P	N	0	N	0	N	N	N								0	50.0
2624	0	0	0	P	P	P	P	0	0	0	0	P	N	0	0	0	P	N	P									0	77.8
2625	0	0	0	P	N	P	P	0	P	0	N	P	0	0	0	N	P	P	0									N	63.6
2626	0	0	N	P	P	P	0	P	0	N	0	N	0	N	0	N	P	0										P	54.5
2627	0	0	P	0	0	P	0	P	P	N	0	P	N	N	N	0												N	50.0
2628	0	0	N	0	0	0	P	P	N	0	0	P	N	N	0													0	42.9
2629	0	0	0	N	0	P	P	N	0	N	N	N	N	N														0	22.2
2630	0	0	0	0	P	P	P	0	0	0	P	0	N															0	80.0
2631	0	0	P	0	P	0	P	N	N	P	0	0	0	0														N	57.1
2633	0	0	P	0	P	0	P	N	N	P	0	0	0	0														0	66.7
% Positive	0.0	33.3	66.7	95.0	92.0	87.0	91.7	65.2	61.1	40.9	70.6	52.9	43.5	10.0	10.0	25.0	35.3	33.3	41.7	20.0	16.7	0.0	22.2	0.0	0.0	0.0	0.0	31.3	

Figure 6-7: Positive and negative samples collected from each calf.
Samples collected from each calf at each time point. A positive sample is denoted by a red “P” while a negative sample is denoted by a blue “N”, “0” indicates that a sample was not collected from that calf on that day. The percentage of positive samples at each time point are shown at the bottom of each column while percentage of positive samples from each individual is shown in the right-hand column.

6.4.3 Speciation of *Cryptosporidium* positive samples by nssm-PCR

All *Cryptosporidium* positive samples were speciated using a nssm-PCR described in section 2.6.2.

6.4.3.1 Adults

Of the 57 *Cryptosporidium* positive samples from the adult cattle, 91.2% (n=52) of the samples were identified as *C. parvum*, 3.5% (n=2) as a mixed infection of *C. parvum* and *C. ryanae,* 1.75% (n=1) as a mixed infection of *C. ryanae* and *C. bovis*, 1.75% (n=1) as a mixed infection of *C. parvum* and *C. andersoni* and 1.75% (n=1) as a mixed infection of *C. parvum, C. ryanae* and *C. bovis* (Figure 6-8).

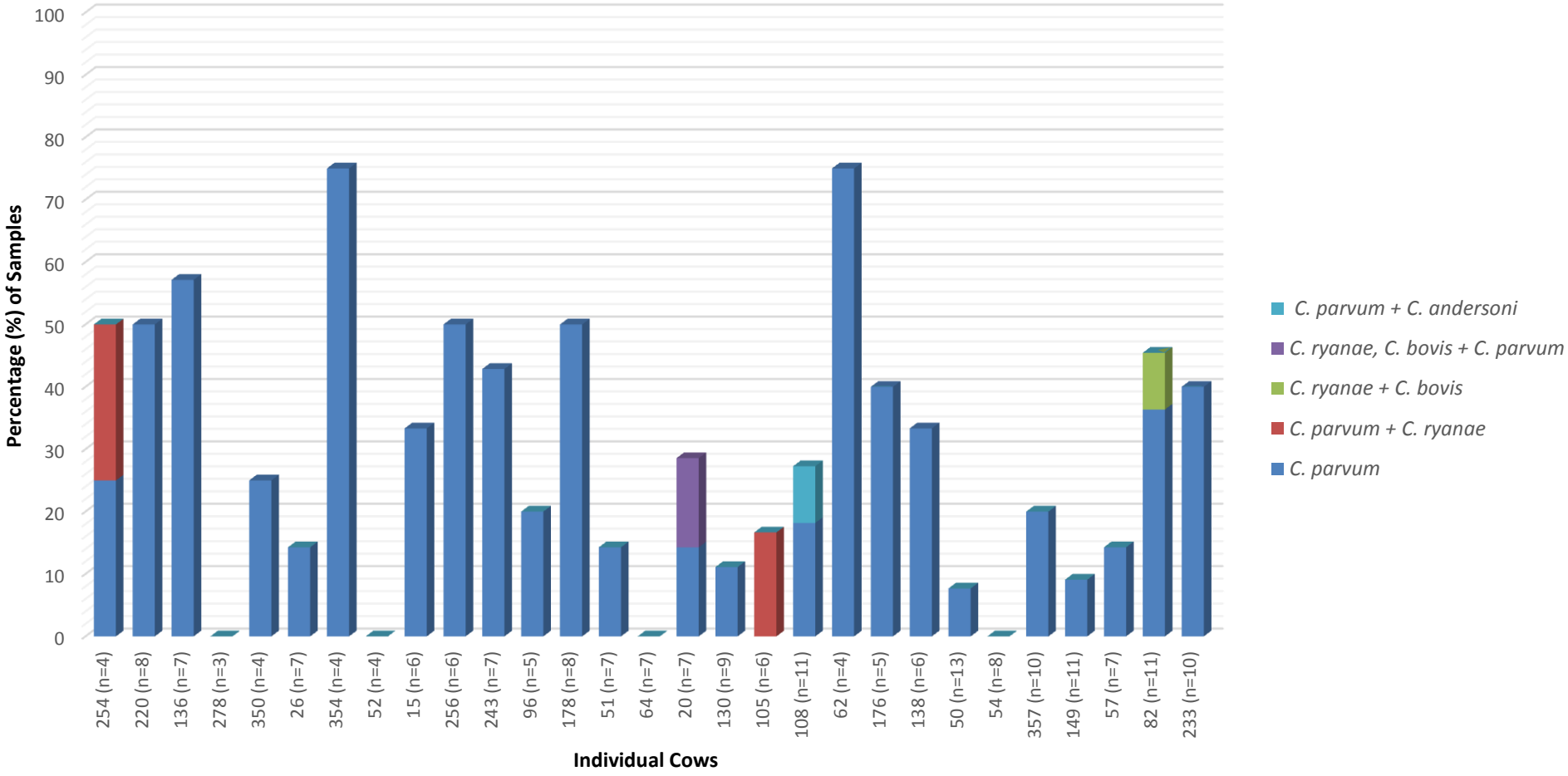


Figure 6-8: Species detected in individual cows.
Percentage of different species detected in positive samples from each individual cow. *C. parvum* was the most frequently detected species in these samples. Four of the cows produced no *Cryptosporidium* positive samples.

To confirm the results of the nssm-PCR a selection of samples (n=19) were sent for sequencing by GATC (see section 2.7), 12 of these failed sequencing (unreadable trace files) the remaining 7 matched the result of the nssm-PCR.

6.4.3.2 Calves

All *Cryptosporidium* positive samples were speciated using the nssm-PCR described in section 2.6.2. All positive samples from calves <9 weeks were *C. parvum* positive. Of the five positive samples from the calves at 6 months old, 20% (n=1) were *C. parvum* positive and 80% (n=4) were mixed infections of *C. parvum* and *C. bovis*.

6.4.4 Subtyping of *C. parvum* by *gp60* PCR

To determine the *gp60* subtypes of the *C. parvum* positive samples collected from both adult cattle and calves a subset of samples were amplified using the *gp60* nested PCR described in section 2.6.3 and sequenced as described in section 2.7.

6.4.4.1 Adults

A small selection (n=32) of *C. parvum* positive samples were subjected to a nested PCR to amplify a fragment of the *gp60* gene. Fifteen of these failed to amplify in the PCR. The remaining seventeen were positive and were sequenced successfully. Four different *gp*60 subtypes were identified; these were IIaA15G2 (n=2), IIaA15G2R1 (n=11), IIaA18G2R1 (n=3) and IIaA19G2R1 (n=1).

6.4.4.2 Calves

A larger selection of *C. parvum* positive samples (n=106) from calves were further analysed using the *gp60* nested PCR, 12 of these failed to amplify. Of the 89 which were amplified successfully, 83 of these were sent for sequencing. Eighty of these returned good trace sequences and two subtypes were identified IIaA19G2R1 (n=75) in the samples from animals <9 weeks of age and IIaA15G2R1 (n=5) in samples from the same animals at 6 months of age (Table 6-3).

Table 6-3: *gp*60 subtypes detected in calves at different ages.

Calf ID	< 1 Weeks	2-4 Weeks	5-8 Weeks	6 Months
2604	IIaA19G2R1	IIaA19G2R1		
2609		IIaA19G2R1	IIaA19G2R1	IIAa15G2R1
2612	IIaA19G2R1			IIAa15G2R1
2613	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	
2617	IIaA19G2R1	IIaA19G2R1	IIaA19G2R1	
2619		IIaA19G2R1		IIaA15G2R1
2620		IIaA19G2R1		IIaA15G2R1
2626		IIaA19G2R1		IIaA15G2R1
2633	IIaA19G2R1	IIaA19G2R1		

6.5 Discussion

6.5.1 Adults

In adult cattle the reported prevalence of *Cryptosporidium* varies markedly between studies (0-71%) (Table 6-4), the most likely reason for this variation is the differing sensitivities of the techniques used to sample and test the faeces. The new and improved techniques developed in this study enabled detection of lower numbers of *Cryptosporidium* oocysts in faeces collected from adult cattle than other methods. Using these techniques it was possible to detect as few as 5 oocysts per gram of faeces which is more sensitive than other methods such as acid fast staining (1000 opg), fluorescent staining (66 opg), although not as sensitive as the gold-standard qPCR which can detect as little as 40 *fg* of DNA (one oocyst) (Hadfield et al., 2011). The increased sensitivity with this method is probably due to a combination of the larger starting sample (50 g) combined with the two methods of concentrating the oocysts. The acid flocculation step removes the majority of fibrous material from the faeces and when followed with salt flotation there is very little debris left in the final pellet. This reduces the amount of inhibitors present in the pellet for DNA extraction and PCR. In this study the samples from the adult cattle were tested by both acid flocculation followed by DNA extraction, and acid flocculation and salt flotation followed by DNA extraction. Using the acid flocculation 5.4% (n=11) samples tested positive compared with 26.8% (n=55) when using both acid flocculation and salt flotation. This indicates that the initial acid flocculation concentration step is not enough to increase the chances of detecting oocysts.

Table 6-4: Summary of studies carried out investigating *Cryptosporidium* in adult cattle.
Table shows the studies carried out to determine prevalence and species of *Cryptosporidium* in adult cattle. The number of animals and samples, methods and results are summarised.

Author(s)	Year	Number of Animals	Number of Samples	Methods Used	%age Positive	Species
(Villacorta et al.)	1991	131	131	Microscopy (Heine's Technique)	1.4%	*C. parvum* (n=2)
(Castro-Hermida et al.)	2006	63	63	Microscopy (Heine's Technique)	8%	No Speciation
(Gow and Waldner)	2006	560	560	Sucrose flotation, Microscopy (DFA)	1%	No Speciation
(Singh et al.)	2006	67	67	Sucrose flotation, Microscopy (mZN)	13.4%	No Speciation
(Lorenzo Lorenzo et al.)	1993	131	131	Microscopy (Heine's Technique)	71.8%	*C. muris** (n=2) *C. parvum* (n=92)
(Scott et al.)	1995	553	553	Two smears of each sample, Microscopy (mZN and FITC to confirm) 50x 5g samples - sucrose flotation, Microscopy (mZN)	62.4%	*C. parvum* (n=345)
(Quilez et al.)	1996	225	225	Formalin-ethyl acetate sedimentation, Microscopy (mZN)	17.8%	*C. parvum* (n=40)
(Bukhari and Smith)	1996	109	109	Water-ether sedimentation, Microscopy (FITC, DAPI)	23.0%	*C. parvum* (n=21) *C. muris** (n=4)
(Esteban and Anderson)	1995	Not Reported	1746	Microscopy (mZN)	8.0%	*C. muris**
(Esteban & Anderson)	1996	1240†	1240	Microscopy (mZN)	0.6-2.6%	*C. muris**
(Atwill et al.)	1998	154	384	Direct fluorescent antibody (DFA) and acid-fast (AF) assays	0.0%	N/A
(Kaneta and Nakai)	1998	512	512	Sucrose flotation, Microscopy (modified Kinyoun acid fast staining), Mouse Bioassay	4.7%	*C. muris** (n=24)
(Atwill et al.)	1999	484	484	Immunofluorescent Microscopy	0.6%	*C. parvum*
(Ares-Mazas)	1999	41	41	Immunofluorescent Microscopy	0.0%	N/A
(Faubert and Litvinsky)	2000	148	Not Reported	500g samples, Sucrose flotation, Microscopy (ZN)	Not Reported	Not Reported
(Fayer et al.)	2000	184	184	10g faecal sample, Filtered, Caesium Chloride Flotation, Fluorescence Microscopy	22.3%	*C. parvum* (n=38) *C. andersoni* (n=3)
(Wade et al.)	2000	998	998	1g Faecal Samples, Sucrose Flotation, Microscopy (bright field and phase contrast)	1.5%	*C. muris** (n=15)
(Enemark et al.)	2002	117	117	Smears, Microscopy (mZN, FITC), one sample was speciated by PCR-RFLP, Mouse Bioassay	40.2%	*C. parvum* (n=12) *C. andersoni* (n=27)

						C. parvum & C. andersoni (n=8)
(Atwill and Pereira)	2003	43	86	2g faecal sample, Microscopy (IMS–DFA)	0.0%	N/A
(Kvac and Vitovec)	2003	96	96	Sucrose Flotation, Microscopy (light), PCR (18S), Sequencing	43.8%	*C. andersoni* (n=42)
(Sturdee et al.)	2003	736	736	Modified Formal-Ether Sedimentation, Microscopy (FITC, mZN)	3.5%	*C. parvum* (n=26)
(McAllister et al.)	2005	669	669	2-3g faeces, Filtered through sponge, Sucrose Flotation, Microscopy (FITC)	29.0%	*C. parvum* *C. muris**
(Fayer et al.)	2006	571	571	15g Faecal Sample, Filtered, Caesium Chloride Flotation, PCR (18S), Sequencing	11.9%	*C. suis* (n=1) *C. parvum* (n=4) Deer-like genotype‡ (n=10) *C. bovis* (n=24) *C. andersoni* (n=29)
(Fayer et al., 2007)	2007	514	514	15g Faecal Sample, Filtered, Caesium Chloride Flotation, PCR (18S), Sequencing	5.7%	*C. parvum* (n=2) *C. bovis* (n=9) *C. andersoni* (n=20)
(Santin et al., 2008)	2008	30	360	15g Faecal Sample, Filtered, Caesium Chloride Flotation, Microscopy (IFA), PCR (18S), Sequencing	2.2%	Deer-like genotype‡ (n=3) *C. andersoni* (n=2)
(Ralston et al., 2010)	2010	567	567	1-5g Faecal Sample, Filtered, Sucrose Flotation, Microscopy (FITC), PCR (18S), Sequencing	3.5%	*C. andersoni* (n=20)
(Wang et al.)	2011	295	295	20g Faecal Sample, Sucrose Flotation, Microscopy (bright field), PCR (18S), Sequencing	1.0%	*C. andersoni* (n=3)
(De Waele et al.)	2012	42	126	2g Faecal Sample, Filtered, Centrifuged, qPCR,	33.3%	*C. parvum* (n=6) *C. bovis* (n=2) *C. andersoni* (n=2)
(Silverlas and Blanco-Penedo)	2013	259	259	1g Faecal Sample, Sodium Chloride flotation, Microscopy (Fluorescence), PCR (18S & GP60)	3.5%	*C. parvum* (n=9)§
(Smith et al.)	2014	2669	2669	10g Faecal Sample, Microscopy (IFAT), PCR (RFLP, GP60), Sequencing	1.9%	*C. andersoni* (n=36)

* Now recognised as *C. andersoni*

† From 5 different herds – no indication is given of the number of cows from each herd

‡ Now recognised as *C. ryanae*

§ Reported in paper as *C. parvum*-like but the single sample which was sequenced was identified as *C. bovis*

It has been reported that the most common species of *Cryptosporidium* to be detected in adult cattle is *C. andersoni* (Fayer et al., 2010a; Feng et al., 2007; Santin et al., 2008; Xiao, 2010). This species is only rarely detected in pre-weaned animals (Amer et al., 2010a; Kvac et al., 2011; Wang et al., 2011b) indicating that adult cattle are not a risk for infection for neonatal calves or that *C. andersoni* is not infective to neonatal calves. Some studies have reported a higher prevalence of *C. andersoni* in young calves, between 11.1-92.9% in calves <35 weeks old on a farm in Bohemia, Czech Republic (Kvac and Vitovec, 2003) and in China *C. andersoni* was found to be the most prevalent species in calves aged 3-11 months (8.70% of samples were *C. andersoni* positive and 2.6% *C. bovis*) (Wang et al., 2011a). Some studies investigating the risk factors associated with cryptosporidiosis in young calves found that calves kept for <72 h with their mothers increased the risk that the calf would suffer from cryptosporidiosis (Quigley, III et al., 1994). Other studies reported the opposite and show that being kept with their dams decreased the likelihood that calves would suffer from cryptosporidiosis (Duranti et al., 2009; Kvac et al., 2006). Most of these risk factor studies did not speciate or subtype the parasites detected in the dams, if, in fact, they even tested the adults for the presence *Cryptosporidium*. Although the present study was not looking at risk factors it did determine the species and subtypes of *Cryptosporidium* being shed by adult cattle and their offspring. We found that the predominant species of *Cryptosporidium* shed by adult cattle on this farm was *C. parvum*. This is unusual though *C. parvum* has been previously reported in adult cattle (Table 6-4). In this study 98.2% (n=56) of the positive samples (n=57) were identified as *C. parvum,* either as a single species or in conjunction with other species. This is a more unusual result when compared with other studies investigating species in adults as most studies show either a low percentage of samples (<20%) from adult cattle are *C. parvum* (Atwill et al., 1999a; De Waele et al., 2012; Enemark et al., 2002; Fayer et al., 2007; Fayer et al., 2006; Quilez et al., 1996; Silverlas and Blanco-Penedo, 2013; Sturdee et al., 2003; Villacorta et al., 1991) or that only a small number of adult cattle (<20%) are positive for any species of *Cryptosporidium* (Atwill et al., 1999a; Atwill and Pereira, 2003; Bukhari and Smith, 1996; Esteban and Anderson, 1995; Kaneta and Nakai, 1998; Quilez et al., 1996; Ralston et al., 2010; Santin et al., 2008; Silverlas and Blanco-Penedo, 2013; Smith et al., 2014; Sturdee et al., 2003; Wang et al., 2011a). Very few

studies show a higher percentage of positive samples from adult cattle (Enemark et al., 2002; Kvac and Vitovec, 2003; Lorenzo Lorenzo et al., 1993; Scott et al., 1995). The results from the present study sit somewhere in the middle at ~30% positive samples. This study was a longitudinal study and it should be noted that if a point prevalence study had been carried out on the same cattle then the percentage of positive samples would have ranged from 19.0% to 66.7% depending on the sampling day. This highlights the underestimation in parasite burden when looking at a single time point.

In this study 86.2% (n=25) of the cows were positive for *Cryptosporidium* at some point throughout the sampling period, again if this had been a point prevalence study a huge underestimation in the percentage of positive animals (0-50%) may have been made. In adult cattle, unlike calves, the prevalence of *Cryptosporidium* seems to vary hugely making it difficult or impossible to apply findings from one study to another. Even within one country or region the results from each study differ. The variation in results may be due to different sensitivities of the techniques used and highlights the need for standardised methodologies for detection of *Cryptosporidium* in adult cattle faeces.

From the results of this study when just taking into account the species of *Cryptosporidium* detected in both the adults and the calves the conclusion may be reached that adult cattle were in fact the source of infection for calves. The majority of positive samples (98.2% (n=56)) from adults were either *C. parvum* or *C. parvum* mixed infections as were all positive samples from calves <9 weeks. In previous studies this is the conclusion that many have made, several of these studies were carried out before we had the knowledge and ability to determine subtypes of these parasites or even before some common species of cattle adapted *Cryptosporidium* had been characterised (Faubert and Litvinsky, 2000; Scott et al., 1995). In this study a single subtype (IIaA19G2R1) was detected in all tested calf samples (n=75) from calves <9 weeks old and four different subtypes (IIaA15G2, IIaA15G2R1, IIaA18G2R1, IIaA19G2R1) were identified in the adults. The most common subtype in the adult cattle was IIaA15G2R1 which was identified in 11 samples from nine cows, this is also the subtype which has been identified in six month old calves in this, and a previous study on the same farm (Chapter 5). This subtype is the most common *gp*60 subtype identified worldwide

in both animal and human hosts (Broglia et al., 2008; Imre and Darabus, 2011). The other subtypes were identified in fewer samples, IIaA15G2 in two samples from two cows, IIaA18G2R1 in three samples from three cows and finally IIaA19G2R1 in a single sample from one cow which was also the most common subtype in calves <9 weeks of age.

One explanation for the single subtype in the young calves is that the one cow which shed IIaA19G2R1 calved early on in the calving season (Figure 6-9). This meant that this subtype was one of the first in the calving shed and could have been picked up by naïve calves. Once a calf is infected with *Cryptosporidium* it amplifies rapidly in the gut (Borowski et al., 2010) and the infected calf can then shed many infective oocysts into the environment where other calves can pick them up and become infected themselves (Nydam et al., 2001). The amplification rate and shedding of huge numbers of oocysts increases the likelihood that other calves will become infected with this subtype rather than another. It is also possible that some calves were infected with more than one subtype but that the calf gut preferentially amplifies one subtype over another or that the techniques used in this study were not sensitive enough to detect another subtype which may be present at a lower level. This is a recognised issue with Sanger sequencing and some researchers are now moving towards a Next Generation Sequencing (NGS) or Multi-Locus Genotyping (MLG) approach. One study which used NGS to study intra-host genetic diversity of *C. parvum* identified 10 different *gp60* alleles in samples which Sanger sequencing had identified only one (Grinberg et al., 2013). In the present study samples from five cows were tested twice and three of these cows were shedding different *gp60* subtypes at each sample point, the remaining two cows shed the same subtype each time. This demonstrates that testing animals once does not give a true and clear picture and also that cattle (and other hosts) are likely to be infected with, or shedding, multiple *Cryptosporidium* species and subtypes at any one time.

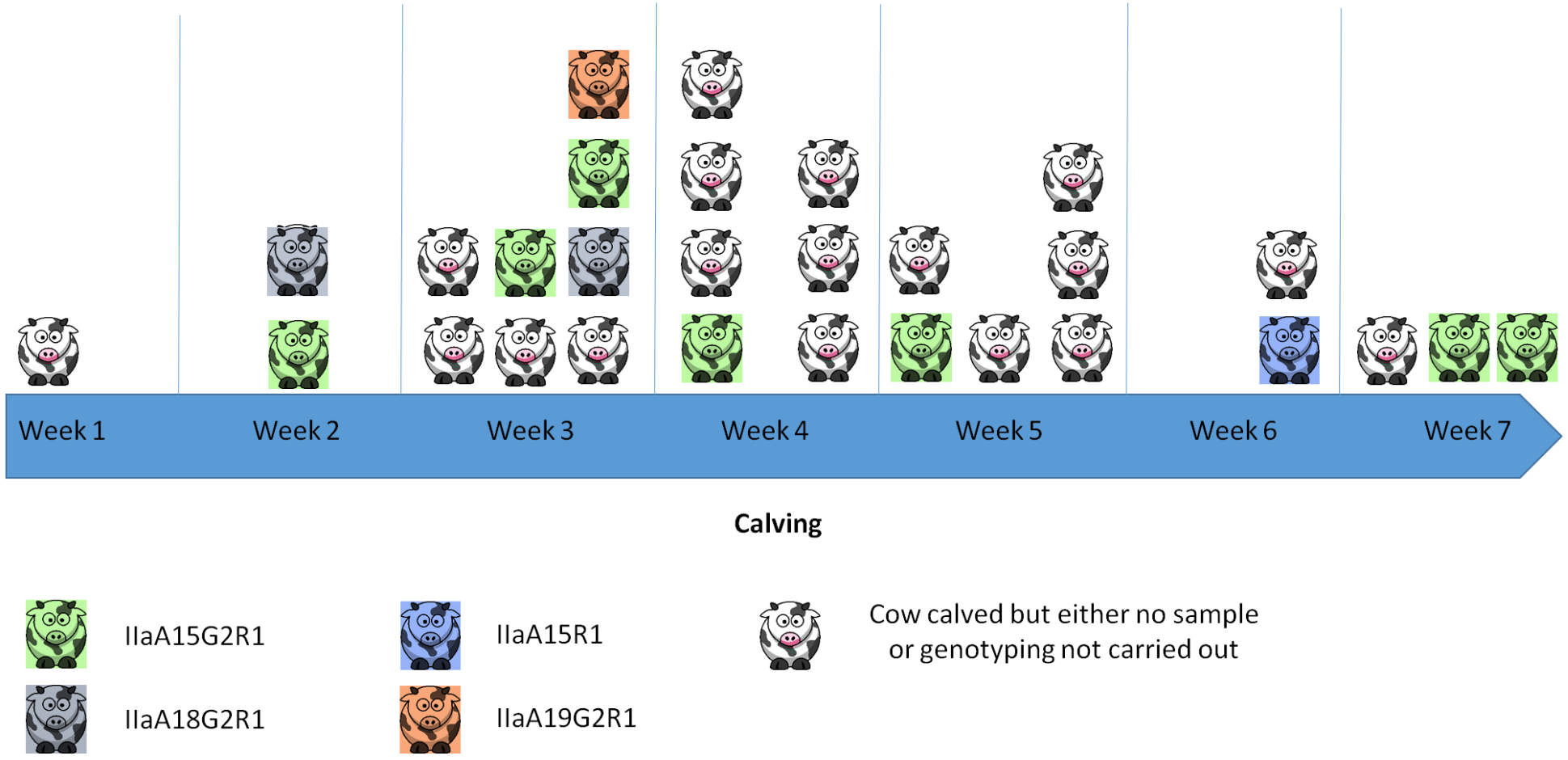


Figure 6-9: Diagrammatic representation of cows calving dates and *C. parvum* subtypes they shed.
Each individual cow is represented by a cow figure and each *gp60* subtype detected is represented by a colour. This diagram represents the subtypes identified in the cows throughout the study, the sample collected at calving may not have been tested.

6.5.2 Calves

6.5.2.1 Comparison with previous calf study

This study was carried out on the same study farm as the study described in Chapter 5. Overall the study design was similar in that a group of calves were sampled and tested for *Cryptosporidium* on a regular basis from birth. The first study (Chapter 5) was carried out between December 2011 and January 2012 and this study approximately a year later from November 2012 until February 2013. The percentage of positive samples in each age group of calves was lower in the second study (Figure 6-10). Generally shedding followed a similar pattern, but with a slightly earlier peak in shedding at week 2 in the second study before tailing off towards the end of week 5. In this study where animals were followed for longer, until 9 weeks of life compared with 6 weeks of life in the first study, there was a secondary peak in positive samples at week 7 (not shown). In both studies the same animals were sampled at 6 months of age, a similar pattern occurs here with the percentage of positive samples in the second study being lower than the percentage in the first study. The overall percentage of positive samples was lower in study two (mean 37.6%) compared to study one (mean 62.9%).

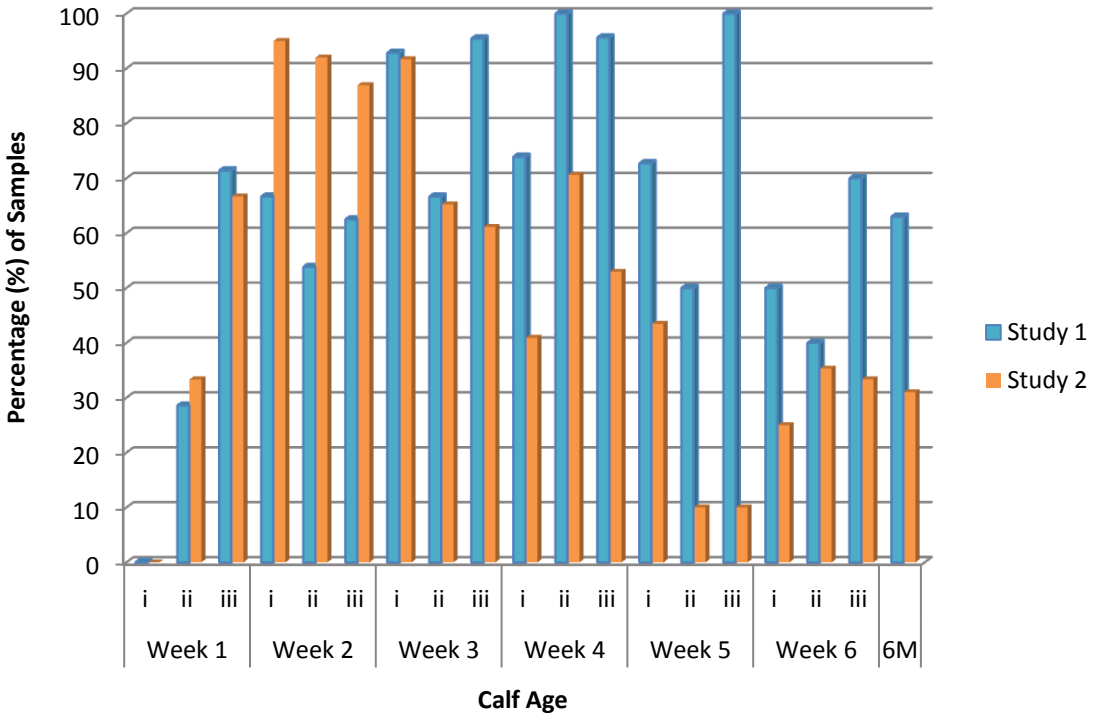


Figure 6-10: Comparison between studies.
Throughout the whole study the prevalence of *Cryptosporidium* was lower in study two compared with study one. The blue bars represent the percentage of positive samples each collection day from calves in study 1 and the orange bars represent the percentage of positive samples collected from calves in study two on each collection day.

Only *C. parvum* was detected in calves in this study whereas *C. bovis*, *C. ryanae* and mixed infections of *C. parvum* and *C. bovis* and *C. ryanae* and *C. bovis* were seen in study one from week three onwards. It is likely that other species or mixed infections were present in study two but that they were missed by both the nssm-PCR and sequencing analysis. As stated previously one failing of PCR amplification when mixed species are present is that the predominant species is often preferentially amplified. Although the nssm-PCR is very effective for detecting mixed infections it is possible that any mixed infections were below the level of detection for this method.

In both studies similar results were found when animals were aged 6 months. In the first study *C. parvum*, *C. bovis* and one mixed infection of *C. bovis* and *C. ryanae* were detected and in the second study *C. parvum* and *C. parvum* and *C. bovis* mixed infections. It is fairly unusual to detect *C. parvum* in older calves but in both studies on this farm *C. parvum* has been the predominant species detected in calves of this age group. Seventy percent (n=7) of the positive

samples for 6 month old calves were *C. parvum* in study one and all positive samples were *C. parvum* positive in study two (80% (n=4) mixed with *C. bovis*).

In both studies a number of samples were subtyped at the *gp60* locus (n=30, study one and n=106, study two) and findings from both studies were similar. All samples tested from calves <9 weeks of age were IIaA19G2R1 and all samples tested from calves 6 months of age were IIaA15G2R1. These results confirm that, on this farm, there are at least two distinct *C. parvum* populations which infect cattle at different times throughout their lives. It is not known if these different subtypes are present in the different age groups as a result of: a local geographical distribution of subtypes on the farm; if something changes in the animal gut to enable the secondary subtype to establish; if the secondary subtype is always present in the animal but at low levels (which the detection methods used here cannot detect) or whether the secondary subtype is, in fact, another species all together. Many of the *Cryptosporidium* species are very similar and only vary genetically by one or two base-pairs at a single locus. Some examples of this would be the two very closely related (genetically 98.50 – 99.70% at certain loci) species which infect humans; *C. parvum*, *C. hominis* but which differ in their epidemiology (Morgan and Thompson, 1999; Morgan-Ryan et al., 2002; Sulaiman et al., 2000). In livestock the species *C. bovis* and *C. xiaoi* are genetically very similar but one is predominantly found in cattle (*C. bovis*) while the other infects sheep (*C. xiaoi*) (Fayer and Santin, 2009; Fayer et al., 2005).

6.6 Conclusion

This study shows that contrary to popular belief adult cattle are a potential source of *Cryptosporidium* infection in calves. Calves are in close contact with faeces from their mother directly at birth. It is very likely that the infection in calves on this farm came from this source as calves show signs of infection and shed oocysts very soon after birth (as few as one day following birth). Further subtyping which was carried out on faecal samples from both calves and dams showed evidence that multiple subtypes of *C. parvum* may be present on a single farm and that these different subtypes may have different clinical manifestations, this is important from both an animal and public health perspective. Although only one adult cow was shown to shed the *C. parvum* subtype which commonly infected calves it is possible that the neonatal calf gut preferentially amplified one subtype over another and therefore the oocysts shed are predominantly the dominant subtype.

Papers in preparation and presentations at conferences

1. Wells, B., **Thomson, S.,** Katzer, F., Development of a sensitive method to extract *Cryptosporidium* oocysts from adult cattle faecal samples. (Submitted)
2. **Thomson, S.,** Jonsson, N., Innes, E.A., Katzer, F., Role of adult cattle in the transmission of *Cryptosporidium*. (In. Prep)
3. The role of adult cattle in the transmission and persistence of *Cryptosporidium* – **5th International *Cryptosporidium* and *Giardia* Conference** – Sweden, 2014
4. Cryptosporidiosis in cattle – **Exchange Visit** – Brazil 2014

Chapter 7: General Discussion

The protozoan parasite *Cryptosporidium* is a common cause of diarrhoea in both human and animal hosts. In livestock hosts the disease caused by *Cryptosporidium* has been associated with reduced weight gain in infected animals (Ralston et al., 2010; Sweeny et al., 2011b) as well as mortality (Blanchard, 2012; Featherstone et al., 2010; Pritchard et al., 2008). There are many different species and genotypes of the parasite (~27 species and >60 genotypes currently recognised) which infect different host species (Chalmers and Katzer, 2013; Fayer, 2010). Some of the species and genotypes are believed to be host specific while others can infect several host species (Fayer, 2010; Ryan et al., 2014). In farm livestock (sheep and cattle) one of the most common causes of neonatal enteritis is *C. parvum* (de Graaf et al., 1999; Mosier and Oberst, 2000), this species of *Cryptosporidium* is also zoonotic and is responsible for ~50% of human cases of cryptosporidiosis in the UK (Chalmers et al., 2009a). Unlike several other pathogens associated with neonatal enteritis in sheep and cattle there is no available vaccine to prevent disease caused by *C. parvum* and treatment options are extremely limited and generally rely on rehydration therapy.

As previously discussed (Chapter 1), cattle may be infected with four different species of *Cryptosporidium* (*C. andersoni, C. ryanae, C. bovis* and *C. parvum*), infection with these species tends to follow an age-related distribution with young calves being infected with *C. parvum*, older calves with *C. bovis* and *C. ryanae* and adult cattle with *C. andersoni* (Fayer et al., 2007; Fayer et al., 2006; Santin et al., 2008; Santin et al., 2004). To date *C. bovis* and *C. ryanae* have not been associated with clinical disease (Fayer et al., 2008; Fayer et al., 2005) and *C. andersoni* has been associated with mild clinical disease (reduction in milk yield and weight-loss) (Kvac and Vitovec, 2003; Ralston et al., 2010). Morphologically these species look very similar, *C. andersoni* is slightly larger and more ovoid than the other three species (Fayer, 2010) which can make diagnosis and species identification difficult when relying on microscopy alone. More advanced methods for species identification generally involve sequence analysis of gene fragments or PCR-RFLP (as discussed in Chapter 1) these

methods can be costly and time consuming and it is also difficult or impossible to identify mixed infections.

The development of immunity to *Cryptosporidium* is poorly understood, it is known that humans and animal hosts can be infected with *Cryptosporidium* more than once demonstrating that the immune response to infection is not particularly strong or long lasting (Okhuysen et al., 1998). Many of the studies into development of immunity and immune responses to *Cryptosporidium* infection have been carried out in murine models. This has given a basic understanding into the possible factors which might play a role in the immune response. However the immune responses in an inbred model may not always be applicable to outbred populations such as humans and livestock hosts. We know that in certain livestock species it appears that as the animals increase in age they become less susceptible to disease (Harp et al., 1990; Kvac et al., 2014; Ortega-Mora and Wright, 1994), it is also known that prior infection can reduce the signs of clinical disease but asymptomatic infection (oocyst shedding) can still occur (Current and Snyder, 1988; Harp et al., 1990; Miller et al., 1990; Ortega-Mora and Wright, 1994). This demonstrates that previous exposure does not provide compete immunity to *Cryptosporidium* but that some level of protection does occur. It is not known if this partial protection can provide protection to infection with another *Cryptosporidium* species or isolate or if the protection is species specific.

The main aims of this PhD were to: assess the development of host resistance to *C. parvum* in experimentally infected lambs; develop a new typing tool to enable easy differentiation of the *Cryptosporidium* species commonly found in cattle; to determine which species and genotypes of *Cryptosporidium* calves became infected with in the first year of life and finally, to assess the role that adult cattle play in the transmission of *Cryptosporidium* (specifically *C. parvum*).

These aims were all achieved with a variety of studies including experimental infections, longitudinal studies and molecular tool design.

A novel technique (Chapter 4) for detection and differentiation of *Cryptosporidium* species commonly found in cattle (*C. andersoni, C. ryanae, C.*

parvum and *C. bovis*) was developed as part of this PhD. This enabled in-depth detailed longitudinal on farm studies to be carried out to investigate the transmission and persistence of *Cryptosporidium* in young calves and adult cattle. The nssm-PCR technique was used extensively throughout this PhD to identify the species of *Cryptosporidium* being shed in the faeces of naturally infected cattle.

The longitudinal study described in Chapter 5 (*Cryptosporidium* species and genotypes found in calves from birth to one year) and the longitudinal study described in Chapter 6 (Transmission of *Cryptosporidium* from dam to calf) confirmed that young calves (<9 weeks) are most commonly infected with *C. parvum* which supports the results of many other studies carried out worldwide (Abu et al., 2013; Amer et al., 2010b; Broglia et al., 2008; Diaz et al., 2010; Follet et al., 2011; Geurden et al., 2007; Huetink et al., 2001; Kvac et al., 2006; Lefay et al., 2000; Rieux et al., 2013; Santin et al., 2004; Silverlas and Blanco-Penedo, 2013; Thompson et al., 2007). The results of Chapter 5 also showed that neonatal calves become infected with *Cryptosporidium* at a very young age, almost immediately at birth, with some calves shedding oocysts at only 2 days of age. This suggested that adult cattle may be the source of infection for newborn calves and lead to the study described in Chapter 6.

The results of both longitudinal studies (Chapters 5 & 6) showed that all calves <9 weeks old on the study farm became infected with *C. parvum* within 2 weeks of life. Infection with *C. parvum* caused severe diarrhoea, inappetence and lethargy in several of the affected animals, others suffered from mild disease only and some animals showed only asymptomatic shedding of oocysts. Shedding of *C. parvum* oocysts occurred for at least 7 weeks PI with peaks in oocysts shedding at 2-3 weeks of age and again at 7 weeks of age (Chapter 5). Clinical signs tended to occur in animals <4 weeks of age and by 6/7 weeks of age many of the infected animals showed no signs of clinical infection though they were still shedding oocysts. In both studies any calves which were still present on the farm at 6 months of age were sampled and their faeces tested again. The results showed that the majority of calves in Chapter 5 (62%) and 32% of calves in Chapter 6 were positive for *Cryptosporidium* and that the most common species detected was *C. parvum*. As discussed previously, this is unusual as it is

generally expected that after initial exposure to the parasite, at a young age, cattle will develop immunity to infection. None of the 6 month old calves were showing any clinical disease at the time of sampling. Subtyping results revealed that the subtype of *C. parvum* identified in the young calves in both studies was IIaA19G2R1 which is an uncommon subtype identified in cattle only a small number of times previously (Couto et al., 2013; Ng et al., 2012; Wielinga et al., 2008; Xiao et al., 2007) while the subtype identified in faeces from 6 month old calves was the "common" subtype IIaA15G2R1 (Broglia et al., 2008; Imre and Darabus, 2011). Perhaps it is possible for cattle to develop immunity to one subtype of *C. parvum* following infection but this immunity may not necessarily protect against further infections with a different subtype. This possibility was tested in the experimental trial described in Chapter 3.

Following the results of Chapter 5 where it was shown that calves can become infected with *Cryptosporidium* almost immediately at birth the second longitudinal study (Chapter 6) focused on the possible transmission of *C. parvum* from dam to calf. Previous studies investigating *Cryptosporidium* infection in adult cattle reported that adult cattle were not believed to be a potential source from which young calves became infected (Atwill and Pereira, 2003; De Waele et al., 2012; Sturdee et al., 2003) although there are two studies which disputed these results (Faubert and Litvinsky, 2000; Kvac et al., 2011). These studies found that adult cattle either shed a species other than *C. parvum* or that they shed such low numbers of oocysts that the authors believed that they were not a significant risk. It is likely that the apparent low levels of *Cryptosporidium* found in adult cattle in previous studies (Table 6-4) could be due to the limitations of the detection methods applied or the number of samples taken. It is known that oocyst shedding and production is not a continuous process and that by taking only a single sample from any individual the overall percentage positivity is underestimated. In previous studies microscopy (with a variety of different staining techniques) was a commonly used method to detect *Cryptosporidium* oocysts. Microscopy is generally a much less sensitive method for the detection of oocysts compared with PCR or qPCR (Chalmers et al., 2011). Only a few of the studies (n=9) used either PCR or qPCR to detect *Cryptosporidium* in adult cattle faeces and many of the studies did not use any prior concentration step before examining the faeces. Faeces from adult

cattle are very different to faeces from young milk fed animals. Faeces from adult animals contain a large amount of fibrous materials and the volume produced is also much greater (~43.1 kg faeces per cow per day) (Weiss, 2015) and so the oocysts may be harder to detect without a concentration step. In the study described in this PhD (Chapter 6) an optimised technique was developed for the concentration and extraction of oocysts from adult cattle faeces which involved an acid flocculation and salt flotation steps (Wells et al., 2015 (to be submitted)).

In this study (Chapter 6) a group of in-calf cattle were sampled 3 × per week from 10 weeks pre-calving until calving (increasing the number of samples collected from each cow) and then the calves born to these cows were sampled 3 × per week for 6-10 weeks. We found that over 80% of the adult cattle involved in this study were shedding *Cryptosporidium* oocysts in their faeces, the majority of positive samples (98.2% (n=56)) were identified as *C. parvum* using the nssm-PCR described in Chapter 4. This finding is unusual as most studies investigating *Cryptosporidium* in adult cattle find only low numbers of positive samples, or animals and the species most commonly detected is *C. andersoni* not *C. parvum*. This is likely to be due to the additional concentration step employed during this study.

Subtyping results of *C. parvum* positive samples from adult cattle showed evidence of four different *C. parvum gp60* subtypes within this farm. One of the subtypes was IIaA19G2R1 which was the only subtype identified in young (<9 week old) calves and another (IIaA15G2R1) which was the only subtype found in older (6 months) calves, these and a further two subtypes (IIaA15G2 and IIaA18G2R1) were also detected in adult cattle on this farm. This age-related distribution of *C. parvum* subtypes in calves on this farm is very interesting; similar results were found in two separate studies in subsequent years on this farm indicating that this age-related distribution of *C. parvum* subtypes occurs frequently on this farm. From other work carried out on this particular farm it is known that the IIaA19G2R1 *C. parvum* subtype has been present and stable since at least 2008 (Gait et al., 2008). None of the other subtypes have been previously detected although other studies have only examined young calves.

As discussed in Chapters 5 & 6 there are several reasons for this apparent distribution of isolates including the possibility that the *C. parvum* isolate detected in 6 month old animals is another species of *Cryptosporidium*. To confirm this it would be necessary to carry out further typing of different loci such as actin, HSP70 and COWP or microsatellite analysis (Hotchkiss et al, 2015 (to be submitted)). Many species of *Cryptosporidium* are very similar to each other at one locus but different at others. Attempts were made to amplify DNA from the parasites from both calves and adults at different loci but these attempts were unsuccessful.

Another reason for the difference in isolates found in calves of different age groups on this farm could be related to changes in the host themselves. It has been reported in previous experimental studies that some host species (pigs and chickens) become more susceptible to certain *Cryptosporidium* species as they increase in age. The authors of these papers (Kvac et al., 2014; Lindsay et al., 1988; Yui et al., 2014) have suggested that this may be due to physiological changes in the gut or the gut flora of the host. It could be that the different isolate found in older cattle is somehow better adapted to replicate in the gut conditions of a fully mature bovine than in the undeveloped gut of a neonatal animal. Other studies examining the subtypes of *C. parvum* in different age groups of cattle on the same farms also found that adult cattle may be carrying different subtypes compared to their calves (Wells et al., 2015) indicating that adult cattle may not be an important source of infection for young calves.

Future work would expand on the findings from these studies and investigate further the possible transmission of *Cryptosporidium* from dam to calf using multiple farms (beef and dairy) with a known history of cryptosporidiosis. Groups of in-calf cows would be sampled regularly, daily if possible, to maximise the number of samples collected. In the study carried out here sample collection occurred 3 × per week but it is not possible to guarantee a faecal sample from each cow at each sampling time. More regular sampling would increase the chances of collecting sufficient numbers of samples from each cow to allow a more complete picture of the shedding dynamics in adult cattle to be formed. In addition to the techniques used in the study described in this thesis it would be beneficial to use a qPCR to quantify the shedding by adult cattle to determine if

shedding of oocysts increases in the approach to calving as suggested by studies on other livestock species (Atwill et al., 1998; Ortega-Mora et al., 1999; Xiao et al., 1994; Ye et al., 2013). This more in-depth approach would give us a more detailed insight into the persistence of the parasite on farms and within animals of differing ages.

Relatively little is understood about the possible differences between subtypes of the same *Cryptosporidium* species. It is known that different subtypes of the same species can vary in their clinical manifestation in infected individuals with some causing more severe disease than others. Some subtypes of *C. hominis* (the human adapted species of *Cryptosporidium*) cause vomiting and diarrhoea in infected people whereas other subtypes cause only diarrhoea (Cama et al., 2008; Cama et al., 2007). It has also been reported that some *C. parvum* isolates appear to be more common in humans (Alves et al., 2003; Cama et al., 2008; Hijjawi et al., 2010) whereas others more commonly infect cattle, sheep or goats (Quilez et al., 2008a; Quilez et al., 2008b). It is not known if these isolates *can* infect other host species or whether they are host-adapted as experimental studies to test this have not been carried out. It does however, demonstrate that even within a species of *Cryptosporidium*, different subtypes can behave differently in different host species. In this thesis a large scale experimental trial was carried out to assess the development of host resistance to distinct *C. parvum* isolates as both homologous and heterologous challenges were performed. Both isolates were known to cause clinical disease in naturally infected calves from two dairy farms in Scotland. The results of this study showed that distinct isolates of *C. parvum* can have different clinical manifestations in infected individuals even when those individuals have received an identical infective dose. The study also demonstrated that host factors play a role in the severity of disease and oocyst shedding as even within groups when animals received the same infective dose of the same isolate there was variation in the outcome of disease with some individuals experiencing more severe or prolonged diarrhoea or increased shedding of oocysts compared to others in the same group. This variation in response has been noted in several other experimental trials involving *Cryptosporidium* and other pathogens (Chappell et al., 1996; Chappell et al., 1999; DuPont et al., 1995; Harp et al., 1990; Moore et al., 2003; Okhuysen et al., 1999; Ortega-Mora and Wright, 1994; Petry et al.,

1995). Although it is clear that there is a difference in the virulence/pathogenicity of isolates there are also host factors which should be investigated further.

In the experimental infection study the results showed that while lambs do not develop complete resistance to disease by 6 weeks of age they do become less susceptible to clinical infection. If lambs are naïve to *Cryptosporidium* and are infected for the first time at 6 weeks of age then they show significantly fewer clinical signs than when lambs are infected at <1 week of age. Another study carried out in 1994, which was similar, demonstrated that lambs become resistant to disease at 8 weeks of age. The lambs in that trial showed no signs of clinical disease though some did shed oocysts when infected at 8 weeks of age. In the trial described in Chapter 3 it was shown that while older lambs exhibited fewer clinical signs of disease than younger lambs they still shed huge numbers of potentially infective oocysts in their faeces. This may be due to the dose of oocysts the animals were given; animals infected at <1 week of age were inoculated with 1×10^6 oocysts while animals infected at 6 weeks of age were inoculated with 5×10^6 oocysts. This increase in infective dose may be the reason why the animals showed asymptomatic shedding of oocysts. Ortega-Mora & Wright (1994) orally infected the lambs in their trial with the same infective dose (1×10^6) at each age group. This may be why, in their study, the older lambs did not show any oocyst shedding. It is possible that if an 8 week old lamb had been challenged with a higher dose as in this trial, that asymptomatic shedding of oocysts would have occurred.

It has been previously demonstrated (Zambriski et al., 2013a; Zambriski et al., 2013b) that an increase in infective dose can lead to an increase in numbers of oocysts shed as well as duration of shedding. In this trial when animals were inoculated with a higher dose, they began shedding oocysts in their faeces much quicker than with the lower dose regardless of any previous exposure. They also shed greater numbers of oocysts following challenge with the higher dose confirming that the infective dose affects the prepatent period and influences oocyst shedding. This means that in a field setting it would be possible for an older animal to become infected with a large dose of *C. parvum* from a young animal and for the older animal to shed huge numbers of oocysts into the

environment while showing no clinical signs of infection itself. This could lead to the infection of a large number of susceptible young animals.

Work which could follow on from this study would involve more detailed investigation into the host factors involved in the development of resistance to disease rather than the differences in the parasite itself. While it is clear that there are differences within subtypes of *Cryptosporidium* parasites of the same species the development of resistance to infection is multifactorial and host factors are very important. Key points to address in future work would be the difference in the innate and adaptive immune responses in young <1 week old animals compared with naïve older animals when infected with *Cryptosporidium* as well as the effect of colostrum. In the experimental trial carried out for this PhD lambs in Group III (BB wk1, BB wk6), IV (BB wk1, LH wk6), V (LH wk1, BB wk6) & VI (LH wk1, LH wk6) all received colostrum from their dams whereas lambs in Group I (BB wk6) & II (LH wk6) received artificial colostrum. Within these two groups (Group I & II) there were still differences in the disease outcome even when given the same colostrum and infective dose. This indicates that other factors are involved which affect the outcome of disease in individuals.

Overall, work carried out for this PhD has enhanced our understanding of the transmission dynamics of the parasite within cattle on the study farm and has indicated that adult cattle may act as a source of infection for young calves although further work is required to confirm this. Improved techniques developed throughout this thesis also advance our ability to quickly and accurately determine the species of *Cryptosporidium* being shed by individual animals as well as increasing the sensitivity of detection methods from adult cattle faeces. These improved techniques have also been used to examine the faeces of other host species such as deer, sheep and rabbits with great success (Wells et al., 2015). The experimental study looking at host resistance has contributed greatly to the field and has answered several questions about the development of immunity as well as the possibility of variation in pathogenicity between isolates of the same *Cryptosporidium* species. This work can be used as a basis to improve our understanding of disease pathogenesis, transmission,

development of host resistance and variation in pathogenicity of different *C. parvum* subtypes.

7.1 PhD Outcomes

The main outcomes from this thesis are:

1) New molecular tool developed to enable speciation and detection of mixed infections of *Cryptosporidium* in cattle faeces (Chapter 4).

2) Adapting (oocyst concentration and purification steps) and improving techniques (DNA extraction) to deal with different sample types can help to ensure accurate results (Chapter 3 & 5).

3) Adult cattle shed more *C. parvum* than previously thought (Chapter 6). The majority of adult cattle in this study shed *C. parvum* at some point, they have potential to be an important source of infection for other susceptible hosts.

4) Adult cattle, though shedding *C. parvum*, may not be an important source of infection for calves (Chapter 6) as they seem to shed different subtypes of *C. parvum*.

5) Cattle can be infected with and shed multiple species and subtypes of *Cryptosporidium* at any one time (Chapter 4, 5 & 6).

6) Clinical disease does not correlate with oocyst shedding (Chapter 3, 5 & 6). Animals can be shedding oocysts without diarrhoea.

7) Cattle do not develop complete sterile immunity to infection with *Cryptosporidium* (Chapter 5 & 6).

8) Lambs do not develop immunity to infection with *Cryptosporidium* in the first six weeks of life although there is a reduction in disease severity at 6 weeks of age (Chapter 3).

9) Infection with one isolate does not provide protection against challenge with the same or a different isolate (Chapter 3).

10) Different *C. parvum* isolates can manifest differently in the host in terms of oocyst output and clinical disease (Chapter 3).

11) Evidence that there is extensive variation in the disease outcome in individual animals in terms of oocyst output and disease manifestation (Chapter 3) indicating that host factors are very important in the outcome of disease.

12) A higher infective dose of *C. parvum* leads to a shorter prepatent period, even in animals with previous exposure (Chapter 3).

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